TO EVALUATE THE COMPARITIVE ORAL BIOAVAILABILITY STUDT OF TROSPIUM CHLORIDE TABLET IN HEALTY HUMAN VOLUNTEERS

Thesis Submitted *to*

The Tamilnadu Dr.M.G.R Medical University, Chennai

In partial fulfillment of the requirements

For the award of the Degree of

***M*ASTER OF PHARMACY**

IN

PHARMACOLOGY

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R.V.S. COLLEGE OF PHARMACY, SULUR, COIMBATORE

SEPTEMBER – 2009

CERTIFICATE

This is to certify that project work entitled '**To evaluate the Comparative oral bioavailability of Trospium Chloride tablet in Human volunteers'** is done by **Mr.V.CHANDRASHEKAR** in partial Fulfillment of the requirement for the award of Master of Pharmacy in Pharmacology Was carried out at WELLQUEST CLINICAL RESEARCH and in R.V.S.College of Pharmaceutical Science, Sulur, Coimbatore.Which is affiliated to **Dr.M.G.R.Medical University, Chennai**, under guidance of **Mr.Nageswara Rao**, and under the guidance of **Mr.R.Suresh** Lecturer in Pharmacology, R V S College of Pharmaceuticel, Science, Sulur , Coimbatore.

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As required by university regulation, I wish to state that this work embodied in this thesis titled **"To Evaluate the Comparative oral bioavailability of Trospium Chloride tablet in Human volunteers"** forms my own **contribution** to The research work carried out under the guidance of **Mr. Suresh** and of **Mr.Nageswara Rao**, This work has not been submitted for any other degree of this or any other university. When ever references have been made to previous work of others, it has been clearly indicated as such and included in the bibliography.

Signature of the Candidate

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Place: Coimbatore

Date: 28-06-2009.

(**Mr.V.CHANDRASHEKAR**)

AIM AND OBJECTIVE

- To evaluate the Comparative oral bioavailability of Trospium Chloride tablet in Human volunteers.

- The study objectives included:
  - Assessment of the bioavailability of test product A while comparing with a reference product B in 6 normal, adult, human subjects under fasting condition. The bioequivalence assessed is to be assessed under following pharmacokinetic Parameters:
    - AUC_{0-t},
    - $AUC_{0-\infty}$,
    - C_{max},
    - T_{max},
    - K_{el}, and
    - $t_{1/2.}$
  - Monitoring of the adverse events and ensure safety of the subjects.

LIST OF ABBREVIATIONS

AE	**Adverse Event**
ANOVA	**Analysis of Variance**
ANDA	**Abbreviated new drug application**
AUC	**Area under the plasma concentration versus time curve**
$AUC_{\_Extrapolated (\%)}$	**Percentage Area Under the Plasma Concentration extrapolated from AUC_{0-t} to $AUC_{0-\infty}$**
$AUC_{0-\infty}$	**The area under plasma concentration versus time curve from time zero to infinity**
AUC_{0-t}	**The area under the plasma concentration versus time curve from time zero to the last measurable concentration**
BA/BE	**Bioavailability / Bioequivalence**
BMI	**Body Mass Index**
CDSCO	**Central Drug Standard Control Organization**
cGMP	**Current good manufacturing practices**
CI	**Clinical Investigator**
C.I.	**Confidence interval**
C_{max}	**Concentration Maximum**
CPB	**Clinical Pharmacokinetics and Biopharmaceutics**
CRA	**Clinical Research Associate**
CRF	**Case Report Form**
CRF,	**Corticotrophin-releasing factor**
C.V.	**Coefficient of variance**
D	**Day**
Dept.	**Department**

CG	**Electro Cardiogram**
ELISA	**Enzyme linked immune sorbant assay**
FDA	**Food and drug administration**
XL	**Extended Release**
GCP	**Good Clinical Practice**
GGTP	**Gamma Glutamyl Transamino Peptidase**
GMP	**Good manufacturing**
HBsAg	**Hepatitis B surface Antigen**
HCT	**Hematocrit**
HCV	**Hepatitis C virus**
HCl	**Hydrochloride**
HIV	**Human Immuno Deficiency Virus**
Hr/Hrs	**Hours**
I.D	**Identity**
ICF	**Informed Consent Form**
ICH	**International Conference on Harmonization**
ICMR	**Indian Council of Medical Research**
IEC	**Independent Ethics Committee**
IP(s)	**Investigational Product(s)**
IL	**Immediate release**
K3EDTA	**Tri-potassium Ethylene Diamine Tetra Acetic acid**
K_{el}	**First order rate constant associated with the terminal (log-linear) portion of the curve**
Kg	**Kilogram**
LLOQ	**Lower limit of quantification**
LSM	**Least Square Means**
LC-MS-MS	**Tandem mass spectroscopy**

M	**Meter**
ME	**Medical Examination**
MR	**Modified Release**
Mg	**Milligram**
ml/mL	**Milliliter**
NA	**Noradrenaline**
ng.hr/mL	**Nanogram. Hour/milliliter**
No.	**Number**
PCV	**Packed Cell Volume**
PI	**Principal Investigator**
PK	**Pharmacokinetic**
PD	**Pharmacodynamic**
QC	**Quality control**
RLD	**Reference listed drug**
RPM	**Rotation per Minute**
SAE	**Serious Adverse event**
SAS	**Statistical Analysis Software**
SGOT	**Serum Glutamate Oxaloacetate Transaminase**
SGPT	**Serum Glutamate Pyruvate Transaminase**
SOP	**Standard Operating Procedure**
TrkB	**kinase-linked receptor**
$T_{1/2}$	**Elimination Half-life**
T_{max}	**Time at Concentration Maximum**
ULOQ	**Upper limit of Quantification**

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CHAPTER 1

INTRODUCTION

The concern today is not just to provide elegant and accurate dosage form but also to ensure that optimum amount of drug reaches the target site. As an example, a specific drug may be prepared as a capsule or tablet, each with different excipients, coating and coloring agents, which may affect the release rate of the drug from the formulation. A thorough background of the fate of drug after its administration, the rate processes to which it is subjected in the body and its behavior after biotransformation are thus very essential in addition to the knowledge about its pharmacodynamics.

Due to the growth of many pharmaceutical industries and due to the post GATT era 2005 there is a tremendous increase in a number of new generic drug products and also there is a tremendous increase in the same type of drug products in the market. This phenomenal growth of the generic pharmaceutical industry and the abundance of multi source products have prompted some questions among many health professionals and scientists regarding the therapeutic equivalency of products, particularly those in certain critical therapeutic categories such as anticonvulsants and cardiovascular.

These multi source pharmaceutical products need to conform to the same standards of quality, efficacy and safety as required of the originator's (Comparator) product. Specifically, the multi source product should be therapeutically equivalent and interchangeable with the comparator (pharmaceutically equivalent or a pharmaceutical alternative) in a pharmacokinetic study with a limited number of subjects in one way of demonstrating therapeutic equivalence without having to perform a clinical trial involving many patients. The purpose of the bioequivalence study is that the bioavailability of the formulations under investigation is shown to be equal. Based on that conclusion therapeutic quality of the formulations are claimed to be identical. The latter means that both the beneficial and side effects are identical and are truly interchangeable. The bioequivalence study thus provides indirect evidence of the efficacy and safety of a multi source drug product. Often this will be the only evidence that the product is safe and efficacious. It is therefore crucial that the bioequivalence study is performed in an appropriate manner .

New formulations of active drug ingredients or therapeutic moieties must be approved prior to marketing by the FDA. In approving a drug product for marketing, the FDA must ensure that the

drug product is safe and effective. Through appropriately designed bioavailability studies, the performance of the formulation used in the clinical trials, provide evidence of safety and efficacy. Bioavailability and bioequivalence studies of drug products and drug product selection have thus emerged as critical issues in pharmacy and medicine during the last three decades.

1.1 DEFINITIONS

Bioavailability: The rate and extent to which the active ingredient or active moiety is absorbed from the drug product and becomes available at the site of action.

Pharmaceutical equivalents: Drug products that contain identical amounts of identical active ingredient, i.e., the same salt or ester of the same therapeutic moiety, in identical dosage forms, but not necessarily containing the same inactive ingredients.

Pharmaceutical alternatives: Drug products that contain the identical therapeutic moiety, or its precursor, but not necessarily in the same amount or dosage form or as the same salt or ester.

Therapeutic equivalents: Drug products are considered to be therapeutic equivalents only if they are pharmaceutical equivalents and if they can be expected to have the same clinical effect and safety profile when administered to patients under the conditions specified in the labeling.

Bioequivalence: The absence of a significant difference in the rate and extent to which the active ingredient or active moiety in Pharmaceutical equivalents or Pharmaceutical alternatives becomes available at the site of action when administered at the same molar dose under similar conditions in an appropriately designed study.

1.2 GENERAL OBJECTIVES OF BIOAVAILABILITY STUDIES

Bioavailability studies are important in the

- Determination of influence of excipients, patient related factors and possible interaction with other drugs on the efficiency of absorption.
- Development of new formulations of the existing drugs.

1.3 HISTORY OF BIOAVAILABILITY

It is commonly observed that there are several formulations of the same drug, in the same dose, in a similar dosage form and meant to be given by the same route. Substitution of one product for another can be made provided they are equally effective therapeutically as the standard required.

In order to ensure the clinical performance of such drug products, bioequivalence studies should be performed.

The concept of bioavailability was initially introduced by Oser 1945, its problem has only recently been recognized and discussed, as a result of controversies involving chloramphenicol, digoxin and phenytoin. A change in formulation caused decreased bioavailability of digoxin in Britain and phenytoin intoxication in Australia.

The development of analytical techniques, during the 1960's, made possible the development of methods sensitive enough to allow quantification of drugs or metabolites, initially in the urine and afterwards in the plasma, making possible the evaluation and the comparison of bioavailability of different formulations, as well as demonstration that significant differences among them could occur.

After the legislation of compulsory drug registration in 1969, which facilitated the introduction of generic drugs in Canadian market, the "Drugs Directorate" of the "Canadian Fasteral Department of Health and Welfare" began to use bioequivalence as a measure to approve the registration of a drug during the 1970's. In 1966, FDA found that out of 4000 formulations available in USA, more than 300 were ineffective. The availability of over 45,000 formulations of 5000 drugs in India, the recent interest in cheap generic formulations and availability of special; long acting formulations have made it imperative for the physician and pharmacist to consider and understand the influence of bioavailability on the therapeutic decisions[4].

In 1977, FDA published the first guidelines for clinical research intending to guarantee data quality and clinical research subject protection. Between 1977 and 1981, new guidelines on Good Clinical Practices were published. In 1988, a consolidated code of Good Clinical Practices (GCP) was published by the FDA.

Good Clinical Practices were also adopted in other countries:

- 1985-Japan and Canada
- 1986-UK
- 1991-Australia and EU
- 1995-Publication of the Code of Good Clinical Practices (GCP) by WHO.

In 1996, the International Conference on Harmonization (ICH) with posterior amendments served as a basis for the conduction of clinical trials, according to similar norms and rules in different countries and in accordance with scientific and ethical standards.

Central Drugs Standard Control Organization suggests that BA/BE data is required to be furnished with applications for the new drugs as required under schedule Y, depending on the type of application being submitted.

WHO guidelines suggest that BE studies should be performed in compliance with the general regulatory requirement and good practices and good laboratory practices guidelines.

Feldman and associates (1982) reported that patients on a high carbohydrate diet have a much long elimination half-life of theophylline due to the reduced metabolic clearance of the drug ($t_{1/2}$ -18.1hr) compared to the patients on a normal diet ($t_{1/2}$ -6.76hr).

FACTORS AFFECTING BIOAVAILABILITY

The systemic absorption of an orally administered drug in a solid dosage form is comprised of three distinct steps:

- Disintegration of the drug product
- Dissolution of the drug in the fluids at the absorption site
- Transfer of drug molecule across the membrane lining the gastrointestinal tract into the systemic circulation .

Any factor that affects any of these steps can alter the drug's bioavailability and thereby it's therapeutic effect. The various factors that can influence the bioavailability of a drug can be broadly classified as dosage form-related or patient-related. Some of these factors are listed in the table below:

TABLE: 1.1 BIOAVAILABILITY FACTORS RELATED TO DOSAGE FORM

PHYSICOCHEMICAL PROPERTIES OF THE DRUG	FORMULATION AND MANUFACTURING VARIABLES
Particle size	Amount of disintegrant
Crystalline structure	Amount of lubricant
Degree of hydration of crystal	Special coatings
Salt or Ester form	Nature of diluent
	Compression force

One of the most important factors that affect the dissolution rate of slowly dissolving substances is the surface area of the dissolving solid. Peak blood levels occurred much faster with the smaller particles than the larger ones, primarily as a result of their faster dissolution rate. Particle size can also have a significant effect on AUC.

There are numerous reports of the effects of formulation and processing variables on the dissolution of active ingredients from drug products: an apparently inert ingredient may affect drug absorption. For example, magnesium stearate, a lubricant, commonly used in tablet and capsule formulations, is water-repellant. Its hydrophobic nature tends to retard drug dissolution by preventing contact between the solid drug and the aqueous GI fluids. Thus, increasing the amount of magnesium stearate in the formulation results in a slower dissolution rate of the drug and decreased bioavailability. The nature of the dosage form itself may have an effect on the drug absorption characteristics .

The effect of food and type of diet on the bioavailability of erythromycin is shown in a study by Welling[12]. The absorption of the antibiotics is significantly reduced when it is administered with food compared with its absorption under fasting conditions. This reduced absorption is primarily a result of degradation of the acid-labile erythromycin due to prolonged retention in the stomach.

Food more usually delays drug absorption without reducing the extent of absorption. This can be of major importance to the patient however, since the onset of drug action can be delayed or even abolished if therapeutic concentrations fail to be achieved in the plasma. Consequently such interactions do warrant a mention. Delay of the onset of therapeutic effect is particularly important regarding analgesics. Willis showed non steroidal anti-inflammatory drugs including aspirin, diclofenac and piroxicam are absorbed more slowly with food than in the fasted state . Though their bioavailability may not be reduced, this is unlikely to reassure the patient whose main concern is to be rid of the pain quickly.

JJ Michnovicz demonstrated that smoking exerts a powerful inducing effect on the 2-hydroxylation pathway of estradiol metabolism, which is likely to decreased bioavailability at estrogen target tissues.

1.4 TYPES OF BA/BE STUDIES

FASTING STUDY

After an overnight fast of at least 10 hours, subjects are made to continue to fast for up to 4 hours after dosing. The drug product is administrated with 240±2 ml of water.

FAST STUDY

After an overnight fast of at least 10 hours, subjects are given a high calorie-high fat breakfast 30 minutes prior to administration of the drug product (dosing). The drug product is administrated with 240±2ml of water. No food is allowed for at least 4 hours post-dose. Water can be allowed as desired except for one hour before and after dosing as per the protocol.

CROSSOVER DESIGN

In these type of study design,

- Each formulation is administered just once to each subject and once in each study period
- All the subjects do not receive the same formulation at the same time; in a given study period, they are administered different formulation.

Period refers to the time period in which a study is performed. A two-period study is a study that is performed on two different days (time periods) separated by a washout period during which most of the drug is eliminated from the body. A sequence refers to the number of different orders in the treatment groups in a study .

1.5 METHOD OF ASSESSING BIOAVAILABILITY

Bioavailability determinations are performed by drug manufacturers to ensure that a given drug product will get to its site of action in an adequate concentration. Bioavailability studies are also carried out to compare the availability of a drug substance from different dosage forms or from the same dosage form produced by different manufactures. Bioavailability can be measured by different methods mentioned below

- Based on Plasma Drug Concentrations
- Based on Urinary Excretion Data
- Based on Acute Pharmacodynamic Effect

- From Well- Controlled Clinical trials
- From Dissolution studies

In all the above mentioned methods Plasma drug Concentration is the direct and reliable method and it is more followed.

BLOOD LEVEL STUDIES

Blood level studies are the most common type of human bioavailability studies and are based on the assumption that there is a direct relationship between the concentration of drug in blood or plasma and the concentration of drug at the site of action. By monitoring the concentration in the blood, it is thus possible to obtain an indirect measure of drug response. Following the administration of a single dose of a medication, blood samples are drawn at specified time intervals and analyzed for drug content. A profile is constructed showing the concentration of drug in blood at the specified time points the samples are taken.

The key parameters to note are:

- AUC
- C_{max}
- T_{max}

SINGLE-DOSE VERSUS MULTIPLE-DOSE

Most bioavailability evaluations are made on the basis of single-dose administration. The argument has been made that single doses are not representative of the actual clinical situation, since in most instances, patients require repeated administration of a drug.

At a steady-state, the amount of drug eliminated from the body during one dosing interval is equal to the available dose (rate in=rate out); therefore, the area under the curve during a dosing interval at steady-state is equal to the total area under the curve obtained when a single dose is administered. This AUC can therefore be used to assess the extent of absorption of the drug, as well as its absolute and relative bioavailability.

Multiple-dose administration has several advantages over single-dose bioavailability studies as well as some limitations.

ADVANTAGES OF MULTIPLE DOSE ADMINISTRATION

- Eliminates the need for a long wash-out period between doses.

- More closely reflects the actual clinical use of the drug.
- Allows blood levels to be measured at the same concentrations encountered therapeutically.
- Because blood levels tend to be higher than in the single-dose method, quantitative determination is easier and more reliable.

LIMITATIONS

- Requires more time to complete.
- More difficult and costly to conduct (requiring prolonged monitoring of subjects).
- Greater exposure of subjects to the test drug increases the potential for adverse reaction.

1.6 PLASMA DRUG CONCENTRATION-TIME PROFILE

A direct relationship exists between the concentration of drug at the bio-phase (site of action) and the concentration of drug in plasma. A typical plasma drug concentration-time curve obtained after a single oral dose of a drug and showing various pharmacokinetic and pharmacodynamic parameters is depicted in fig.1.1. Such a profile can be obtained by measuring the concentration of drug in plasma samples taken at various intervals of time after administration of dosage form and plotting the concentration of drug in plasma (Y-axis) versus the corresponding time at which the plasma sample was collected (X-axis).

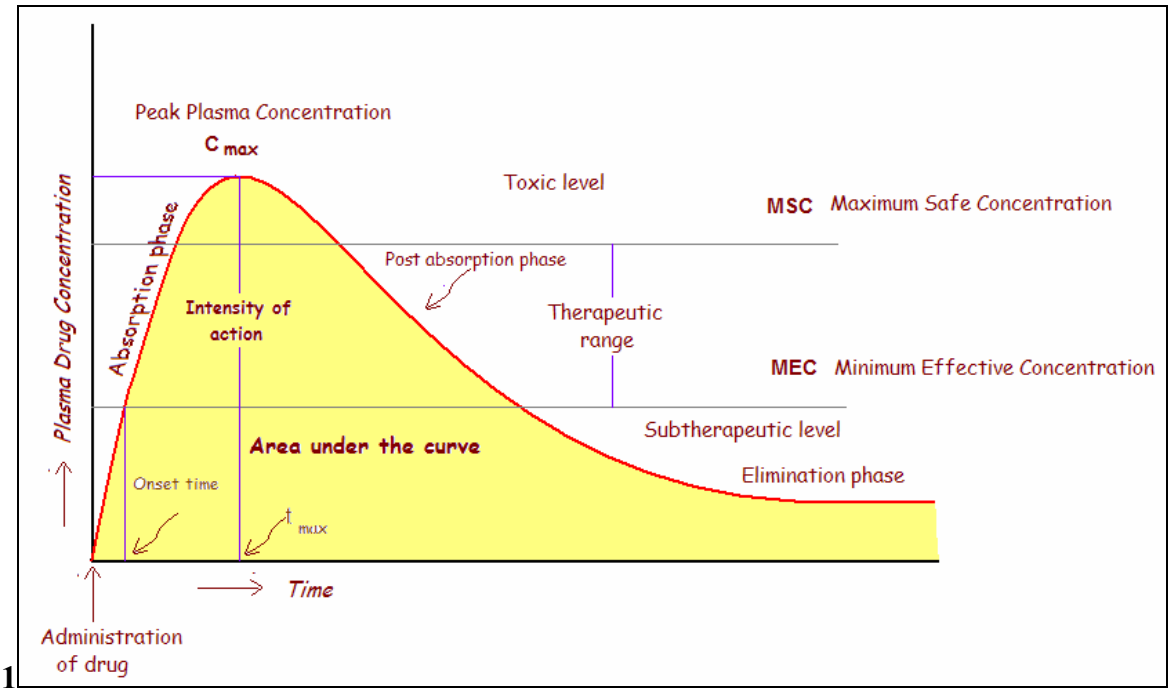


Fig: 1.1 Typical plasma concentration-time profile showing pharmacokinetic and pharmacodynamic parameters

The main pharmacokinetic parameters used for the bioavailability evaluation are:

1.6.1. Peak Plasma Concentration (C_{max})

The point of maximum concentration of drug in plasma is called as the peak and the concentration of drug at peak is known as peak plasma concentration. It is also called as peak height concentration and maximum drug concentration. C_{max} is expressed in mcg/mL. The peak level depends upon the administered dose and rate of absorption and elimination. The peak represents the point of time when absorption rate equals elimination rate of the drug. The portion of curve to the left of peak represents absorption phase i.e. when the rate of absorption is greater than the rate of elimination. The section of curve to the right of peak generally represents elimination phase i.e. when the rate of elimination exceeds the rate of absorption.

.6.2. Time of Peak Concentration (T_{max})

The time for drug to reach peak concentration in plasma (after extravascular administration) is called as the time of peak concentration. It is expressed in hours and is useful in estimating the rate of absorption. Onset time and onset of action are dependant upon t_{max}.

1.6.3. Area under the Curve (AUC)

It represents the total in grated area under the plasma level profile and expresses the total amount of drug that comes into the systemic circulation after its administration. AUC is expressed in mcg/mL X hours. It is the most important parameter in evaluating the bioavailability of a drug from its dosage form as it represents the extent of absorption.

1.6.4. Elimination Rate Constant (K_{el})

The decline in plasma drug concentration is only due to elimination of drug from the body (and not due to distribution), the phase being called as elimination phase. Elimination phase can be characterized by 3 parameters-elimination rate constant, elimination half-life and clearance.

1.6.5. Elimination Half-Life ($T_{½}$)

It is also called as biological half-life, it is the oldest and the best known of all pharmacokinetic parameters and was once considered as the most important characteristic of a drug. It is defined as the time taken for the amount of drug in the body as well as plasma concentration to decline by one-half or 50% its initial value. It is expressed in hours or minutes.

Two drugs show the same response if they reach the minimum effective concentration (MEC) at the same time and are considered to be bioequivalence if the mean AUC and mean C_{max} of two products do not differ by more than 20%; the products are then considered to have same clinical effect in patients. The various pharmacodynamic parameters which influence the above mentioned pharmacokinetic parameters are:

Minimum Effective Concentration (MEC)

It is defined as the minimum concentration of drug in plasma required to produce the therapeutic effect. It reflects the minimum concentration of the drug at the receptor site to elicit the desired pharmacologic response. The concentration of drug below MEC is said to be in the sub therapeutic level.

Maximum Safe Concentration (MSC)

Also called as minimum toxic concentration (MTC), it is the concentration of the drug in plasma above which adverse or unwanted effects are precipitated. Concentration of drug above MSC is said to be in the toxic level.

Onset of Action

The beginning of pharmacologic response is called as onset of action. It occurs when the plasma drug concentration exceeds the required MEC.

Onset Time

It is the time required for the drug to start producing pharmacologic response. It corresponds to the time of plasma concentration to reach MEC after administration of drug.

Duration of Action

The time period for which the plasma concentration of drug remains above the MEC level is called as duration of drug action.

Therapeutic Range

The drug concentration between MEC and MSC represents the Therapeutic range.

1.7 LIQUID CHROMATOGRAPHY- TANDEM MASS SPECTROMETRY (LC-MS/MS)

Mass Spectrometry (MS) combined with the separation power of chromatography has revolutionized the way we do chemical analysis today. LC-MS is a powerful technique used for many applications which has very high sensitivity and specificity. Generally its application is oriented towards the specific detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture). The API source can operate using

- Electro spray (ESI),
- Nanospray (NSI) or
- Atmospheric pressure chemical ionization (APCI) techniques.

In ESI, the sample solution is sprayed in a fine mist of charged droplets containing sample ions by application of a large negative or positive voltage (typically ±4.5 to ±5 kV). A flow of nitrogen drying gas is directed at droplets and individual positive or negative ions are produced. ESI accommodates a liquid flow of 1 mL/min to 1 mL/min. This ionization technique is very

suitable for the analysis of polar, thermally labile molecules such as drugs, DNA, RNA, sugars, peptides, and proteins.

NSI is essentially ESI operating at very low liquid flow rates of 100 nL/ml to several micro-liters per minute in static or dynamic modes. Static NSI is a self-sustaining direct infusion of a low volume of sample over an extended period of time (1 to 5 ml can be sprayed over 30 to 60 min) and allows an investigation of a sample in MS and MS modes. There is no LC attachment and investigation and maintanance of the spray conditions is assisted with a constant gas backpressure. Dynamic NSI allows connectivity to micro and nano LC columns adding the advantage of a chromatographic separation. The technique provides a tool for the most sensitive analytical challenges.

1.8 BIOANALYTICAL METHOD

A bio-analytical method is a set of procedure involved in the collection, processing, storage and analysis of a biological matrix for a chemical compound. The availability of selective and sensitive bio-analytical methods is a prerequisite for the generation of reliable data on pharmacokinetics, bioavailability and bioequivalence of drugs. These methods should allow the quantification of drugs and their metabolites in biological matrices, e.g. plasma, urine, and cerebrospinal fluid and must be validated with respect to their reliability for the intended use. Bio-analysis frequently involves the measurement of very low analyte concentrations in complex and potentially variable matrices. Quantification of drugs and their metabolites in biological matrices currently is one of the most important applications of LC-MS.

PURPOSE

Bio-analytical method intended for the estimation of drugs in biological fluids and Developing suitable analytical method for,

- Identification and Quantification of different drugs and their metabolites from biological fluids.
- Uses of detection techniques that is highly sensitive and specific for the quantification of drugs .

1.9 METHOD DEVELOPMENT

In the past years, method development for quantitative bio-analysis using LC-MS has changed significantly. While in the past most attention was given to finding the optimum mobile phase composition for the ionization technique selected, nowadays it is realized that parameters related to sample pretreatment, chromatography, analyte ionization and mass spectrometric analysis are all strongly interrelated. One cannot change one parameter without influencing many others. The choice between electro spray ionization (ESI), Atmospheric pressure chemical ionization (APCI) or another ionization technique should be made on the basis of analytical results with spiked pretreated samples. Only in this way matrix effects can be properly evaluated. In addition, optimum solvent conditions in analyte ionization are generally less important that achieving an appropriate chromatographic separation .

SAMPLE PRETREATMENT

A wide variety of sample pretreatment methods are applied in quantitative bio-analysis using LC-MS. In general, the three major goals of sample pretreatment are,

- Removal of unwanted matrix components, which may interfere in the LC-MS analysis, e.g., compounds with high surface activity and nonvolatile compounds like proteins and salts.
- Pre concentrate or enrich the analyte to improve the limits of quantitation.
  Exchange the analyte into a (more) favorable solvent composition.

TECHNIQUES

PROTEIN PRECIPITATION

Protein precipitation is a routine, high throughput bio-analytical sample preparation technique. It is used to remove proteins from biological fluid samples prior to the analysis of drugs and their metabolites by LC-MS/MS. The technique has wide applicability in bio-analysis from discovery support through to clinical studies. Historically, protein precipitation has been carried out in vials or collection plates, followed by centrifugation. Protein precipitation by 96-well filtration has more recently been used as a high throughput, easy to automate and alternative to the traditional approach. However, most filter plates require the plasma sample to be dispensed before the

precipitating solvent is added. This approach can lead to filtrate breakthrough before precipitation is complete, resulting in cloudy extracts and blocked wells.

LIQUID-LIQUID EXTRACTION (LLE)

LLE is the direct extraction of biological material with a water immiscible solvent. The analyte is isolated by partitioning between the organic phase and aqueous phase. Partition or distribution of a drug between two immiscible liquid phases can be expressed in term of distribution coefficient. Partition coefficient is constant only for a particular solute at specific temperature and pair of solvent used. Liquid-liquid extraction is probably the most widely used techniques because the anaylte can remove a drug or metabolite from larger concentration of endogenous material that might interfere with the final analytical determination. The technique is simple, rapid and has a relatively small cost factor per sample when compared to other methods. The extraction containing drug can be evaporated to dryness and the residue can be re-dissolved in a smaller volume of a more appropriate solvent (mobile phase). Near quantitative recoveries (90 %) of most drug can be obtained through multiple or continuous extraction.

SOLID-PHASE EXTRACTION

In solid phase extraction the analyte is retained on solid phase sorbent while sample passes through, followed by elution of analyte with appropriate solvent. A typical SPE sorbent consists of 40-60 μm silica particles which are bonded with hydrocarbon phase. This bonding is achieved by reaction of chlorosilane with the hydroxyl group of silica gel to form silicon oxygen-silicon link.

1.10 VALIDATION OF BIOANALYTICAL METHOD

Linearity and Standard Curve

A standard curve is the mathematical functional relationship between instrument response and known concentration of the analyte. Concentration of the chemical reference standard (CRS) substance should be justified on the basis of the expected range. A standard curve should include blank sample (without internal standard), zero sample (with internal standard) and six to eight non-zero samples covering the expected range, including LLOQ .

Lower Limit of Quantification (LLOQ)

The lowest standards on the calibration curve should be accepted as the limit of quantification if the following conditions are met,

- The analyte response at the LLOQ should be at least 5 times the response compared to blank response.
- Analyte peak (response) should be identifiable, discrete and reproducible with a precision of 20 % and accuracy of 80-102 %. Normally it is taken as the 10% of C_{max} of the drug.

Calibration/ Standard curve/Concentration-Response

The simplest model that adequately describes the concentration response relationship should be used. Selection of weighting and use of a complex regression equation should be justified. The following condition should be met in developing a calibration curve,

- 20 % deviation of the LLOQ from nominal concentration.
- 15 % deviation of standards other than LLOQ from nominal concentration.

SPECIFICITY

It is the ability of analytical methods to differentiate and quantify the analyte in the presence of other sample components.

Analysis of blank samples for appropriate biological matrix was obtained from at least 6 individual sources. Each blank sample should be tested for interference and selectivity should be ensured at the lower limit of quantification (LLOQ).

ACCURACY

The accuracy describes the closeness of mean test results obtained by the method to the true value of the analyte.

PRECISION

The precision describes the closeness of individual measurements of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix.

Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, inter-batch precision or

repeatability, which measures precision with time, and may involve different analysts, equipment, reagents and laboratories.

It should include minimum three levels of the expected concentration range and minimum five replicate at each level.

RECOVERY

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix compared to the detector response obtained for the true concentration of the pure authentic standards.

Recovery pertains to the extraction efficacy of an analytical method within the limit of variability. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium and high) with un-extracted standards that represents 100% .

STABILITY

The stability of an analyte in a biological matrix is a function of storage conditions, the chemical properties of the drug, the matrix and the container system. Stability procedure should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process.

a. Freeze and Thaw Stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze-thaw cycle should be repeated two more times, then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at -70^0C during the three freeze and thaw cycles.

b. Short-Term Temperature Stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

c. Long-Term Stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

d. Stock Solution Stability

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions .

1.11. CALCULATION OF PHARMACOKINETIC PARAMETERS

Calculation of AUC_{0-t}

The Area under curve (AUC_{0-t}) is calculated by taking the average of two subsequent plasma concentrations (C_i and C_{i-1}) and multiplying that average by the time difference between the consecutive measuring points (t_i and t_{i-1})

All these outcomes are then summed to render the AUC from 0 to the last measuring time point. This approach is called the linear trapezoidal approach.

$$AUC_{0-t} = \sum_{i=1}^{t} \left(\frac{C_i + C_{i-1}}{2} \right) (t_i - t_{i-1})$$

Calculation of C_{max}

Calculation of C_{max} is very simple; the highest concentration value is C_{max}

Calculation of T_{max}

The time point corresponding to C_{max} is the value of $t_{max.}$

Calculation of K_{el} and $T_{½}$

The calculation of $\mathbf{K_{el}}$ is an essential part of any bioequivalence study. Elimination is a first order process and a natural log (ln) - transformation makes it possible to draw a straight line through

the elimination phase. The slope of the regression line is equivalent to K_{el} or the elimination constant.

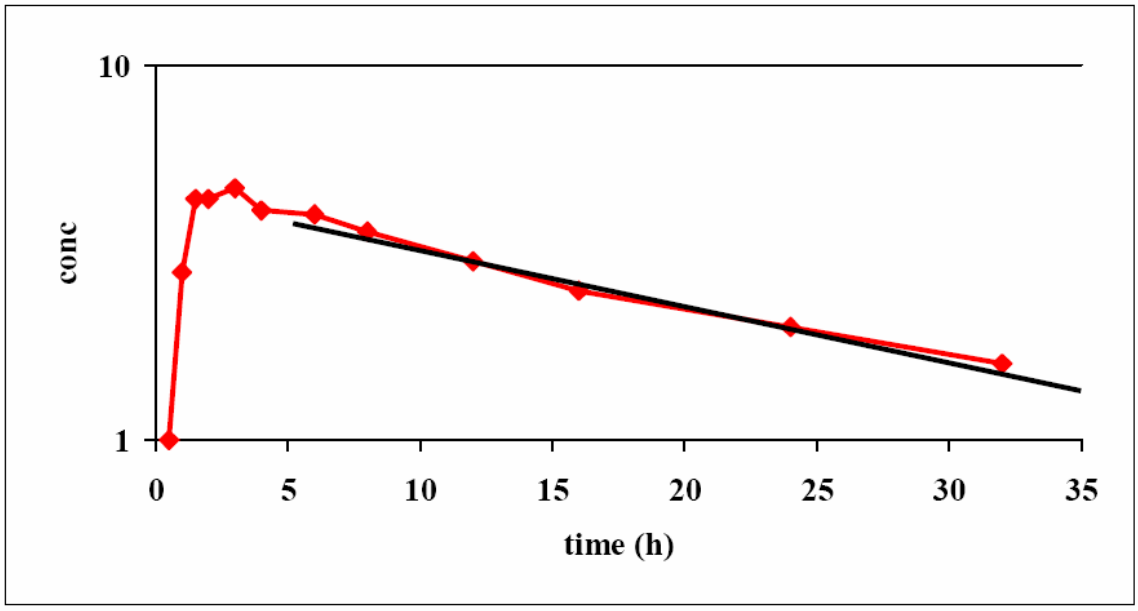


Fig: 1.2 Natural log transformed plasma concentration time-curve showing a regression line through the elimination phase.

The calculation of the half-life is rather simple. One simply divides 0.693 by K_{el} to obtain $t_{½}$. The relationship between $t_{½}$ and K_{el} : $t_{½} = 0.5\ K_{el}$. The term 0.693 is derived from ln(0.5) = 0.693 (ignoring signs). K_{el} describes the lowering of ln transformed plasma concentration per time unit to obtain correct estimate of $t_{½}$.

$$T_{½} = \frac{0.693}{K_{el}}$$

Calculation of AUC_{tot}

The next step in process is to extend the plasma concentration time profile to infinity to obtain $AUC_{tot.}$ The latter parameter is an estimate of total mass of drug present in blood and also serves as a guide for adequate sampling.

To do so K_{el} is of course the most logical parameter, next to the last plasma concentration. K_{el} describes the loss of drug per time unit (/h) so division by of C_{last} (mg/l) results in ameasure with the unit mg/l*h, which is an unit for AUC. The outcome of this calculation is the AUC from t_{last} to infinity (AUC_{tot}), to obtain AUC_{tot} ,$AUC_{0\text{-}t}$ and AUC_{tot} is added

$$AUC_{0-\infty} = AUC_{0-t} + \frac{C_{last}}{K_{el}}$$

1.12 STATISTICAL CRITERIA

After a bioequivalence study is conducted and the appropriate parameters are determined, the pharmacokinetic data must be examined according to a set of predetermined criteria to confirm or refute the bio-equivalency of the test and reference formulations. That is, one must determine whether the test and reference products differ within a predefined level of statistical significance. Since the statistical outcome of a bioequivalence study is the primary basis of the decision for or against therapeutic equivalence of two products, it is critically important that the experimental data be analyzed by an appropriate statistical test.

In the early 1970s, bioequivalence was usually determined only on the basis of mean data. Mean AUC and C_{max} values for the generic product had to be within +20% of those of the reference (innovator) product . Although the 20% value was somewhat arbitrary, it was felt that for most drugs, a 20% change in the dose would not result in significant differences in the clinical response to drugs.

A relatively common misconception is that current regulatory standards still allow this difference of 20% in the means of the pharmacokinetic variables (C_{max} and AUC) of the test and reference formulations. The FDA's statistical criteria for approval of generic drugs now requires the application of confidence limits to the mean data, using an analysis known as the two one-sided tests procedure. This change came about as a result of the conclusion of the FDA Bioequivalence Task Force in 1986 that the use of a 90% confidence interval based on the two one-sided t-tests approach was the best available method for evaluating bio-equivalence.

Westlake was the first to suggest the use of confidence intervals as a means of testing for bioequivalence. Recognizing that no two products will result in identical blood-level profiles, and that there will be differences in mean values between products, Westlake pointed out that the critical issue was to determine how large those differences could be before doubts as to therapeutic equivalence arose. A test formulation was considered to be bioequivalent to a reference formulation, if

$$0.8 < \frac{AUC_{test}}{AUC_{ref}} < 1.2 \text{ and } 0.8 < \frac{Cp_{max_{test}}}{Cp_{max_{ref}}} < 1.2 \ .$$

And by this procedure, if test and reference products were not bioequivalent (i.e. means differed by more than 20%), there was a 5% chance of concluding that they are bioequivalent.

The current FDA guidelines are that two formulations whose rate and extent of absorption differ by -20%/+25% or less are generally considered bio-equivalent. In order to verify that the -20%/+25% rule is satisfied, the two one-sided statistical tests are carried out: one test verifies that the bioavailability of the test product is not too low and the other to show that it is not too high. The current practice is to carry out the two one-sided tests at the 0.05 level of significance. Computationally, the two one-sided tests are carried out by computing a 90% confidence interval. For approval of an ANDA, a generic manufacturer must show that the 90% confidence interval for the ratio of the mean response (usually AUC and C_{max}) of its product to that of the innovator is within the limits of 0.8 to 1.25.

Since these tests are carried out at the 0.05 level of significance, there is no more than a 5% chance that they will be approved as equivalent if they differ by as much or more than is allowed by the equivalence criteria (-20%/+25%). Since this test requires that the 90% confidence interval of the difference between the means be within a range of -20%/+25%, it is more stringent than simply requiring the comparison of the test and reference products' AUC and C_{max} to be within the 80 to 125% range. If the mean response of the generic product in the study population is near 20% below or 25% above the innovator mean, one or both of the confidence limits will fall outside the acceptable range and the product will fail the bioequivalence test. Thus, the confidence interval requirement ensures that the difference in mean values for AUC and C_{max} will actually be less than -20%/ +25%. It should be pointed out that the standards vary among drugs and drug classes. For example, antipsychotic agents may fall within a 30% variation and anti-arrhythmic agents may be allowed a 25% variation.

The actual differences between brand and generic products observed in bioequivalence studies have been reported to be small. The FDA has stated that for post-1962 drugs approved over a two-year period under the Waxman-Hatch bill (1984), the mean bioavailability difference between the generic and pioneer products has been about 3.5%. In addition, 80% of the generic drugs approved by the FDA between 1984 and 1986 differed from the innovator products by an observed difference of only +5%. Such differences are small when compared to other variables of drug therapy and would not be expected to produce clinically observable differences in patient response.

CHAPTER -2

OBJECTIVES OF THE PRESENT STUDY

i. To evaluate the comparative oral bioavailability of Trospium Chloride tablet (each containing Trospium chloride 20 mg) with that of reference product SANCTURA (each containing Trospium chloride 20 mg) of in healthy, adult, male, human subjects under fast conditions.

ii. To develop a bio-analytical method and to validate the Trospium Chloride content in human Plasma using the LC-MS/MS method.

iii. To find out whether the test and reference products are biologically equivalent or not.

CHAPTER-3

REVIEW OF LITERATURE

Pietzko et al., (1994) have reported on the Influences of trospium chloride and oxybutynin on quantitative EEG in healthy volunteers. And also they have examined the possibility of these two drugs produce changes in central nervous electrical activity in an open, prospective, phase I study involving 12 volunteers. The biological activity of both drugs was ascertained by continuous simultaneous recording of the heart rate. A decrease in heart rate was detected after oral administration of oxybutynin, and an increase was seen after i.v. administration of trospium chloride. The results suggest that trospium chloride is less likely to produce central nervous adverse effects than to oxybutynin..

Norman Zinner et al., (2004) have identified that the trospium chloride improves overactive bladder symptoms by a multi-center phase III trial and reported that it has no known drug-drug interactions, an advantage for patients taking many medications. Because these qualities may provide added benefit when treating patients with symptoms associated with overactive bladder and urge incontinence, and studied the effectiveness of trospium in treating these conditions. Trospium significantly decreased average frequency of toilet voids and urge incontinent episodes compared to placebo. It significantly increased average volume per void, and decreased average urge severity and daytime frequency. All effects occurred by week 1 and all were sustained throughout the study.

David ., (2003). have evaluated the clinical pharmacokinetics of drugs used to treat urge incontinence. Unfortunately, the pharmacological activity of the reagents is not limited to the urinary tract, leading to systemic adverse effects that often promote non adherence. Although

the pharmacokinetics of flavoxate, propantheline, scopolamine, imipramine/ desipramine, trospium chloride and propiverine are also reviewed here, only for oxybutynin and tolterodine are there adequate efficacy/tolerability data to support their use in urge in continence. Tolterodine is metabolised via CYP2D6 to the active metabolite 5-hydroxymethyl-tolterodine and via CYP3A to *N*-dealkylated metabolites. Urinary excretion of parent compound plays a minor role in drug disposition. In addition, the use of alternative formulations may also facilitate adherence, not only by reducing the frequency of drug administration but also by enhancing tolerability by altering the proportions of parent compound and active metabolite in the blood.

Halaska et al., (2003). Have carried out controlled, double-blind, multi-centre clinical trial to investigate long-term tolerability and efficacy of trospium chloride in patients with detrusor instability. The trial comprised a total of 358 patients with urge syndrome or urge incontinence. At intervals of 4–8 weeks, patients were physically examined with measurements of blood pressure and pulse rate, were questioned about any adverse events, checked for compliance and underwent relevant laboratory tests. The main symptom encountered in both treatment group was dryness of the mouth. For patients on trospium chloride, the estimated risk of an unexpected adverse event was 0.027 per patient per week for all adverse events and 0.009 for dryness of the mouth, resulting in a considerably lower risk during treatment given with trospium chloride than with oxybutynin. An overall assessment for each of the drugs reveals a comparable efficacy level and a better benefit risk ratio for trospium chloride than for oxybutynin due to better tolerability.

Franqoise et al., (2003) have validated the liquid chromatographic and gas chromatographic Methods. Applications to pharmacokinetics validation of analytical methods are important for the generation of data for bioavailability, bioequivalence and pharmacokinetic studies. It is essential to use well defined and fully validated analytical methods to obtain reliable result s that can be satisfactorily interpreted. This manuscript is intended to provide guiding principles for the evaluation of a method's overall performance. For this purpose, all of the variables of the method are considered, including sampling procedure, sample preparation, chromatographic separation, detection and data evaluation. The criteria considered are as

follows; stability, selectivity, limits of quantification and of detection, accuracy, precision, linearity, recovery and ruggedness. Models used for analytical calibration curves are explained in term of validity and limitations, along with a presentation of the most common statistical considerations used to validate the model..

Konstanze et al., (2003) have carried the randomized, double-Blind Study of the Effects of Oxybutynin, Tolterodine, Trospium Chloride and placebo on sleep in healthy young volunteers. The central nervous effects of oral anti cholinergic may limit the success of incontinence therapy and patient compliance. Only a few studies investigating this topic are available. This study was conducted to determine whether oral anti-cholinergics alter sleep and psychometric test parameters. The proposed design is randomized, double-blind, crossover, placebo-controlled study. study participants are 24 healthy volunteers without sleep related and the results of rapid eye movement sleep was the primary parameter of polysomnography. The REM sleep for oxybutynin was significantly lower than that for trospium chloride and lower than that for placebo The number combination test , the primary parameter of cognitive function, and the d2 test did not reveal any differences in reaction time. With regard to the other sleep parameters, the REM latency for oxybutynin was clearly higher than that for placebo, trospium chloride and tolterodine..

G.lose et al., (2001) have evaluated the intra-vesical oxybutynin for treating incontinence resulting from an overactive detrusor .The use of oral oxybutynin for treating bladder hyperactivity is well documented but despite its clinical efficacy, some patients cannot tolerate the systemic side-effects caused by the oral administration of oxybutynin. The intra vesicle administration of oxybutynin is an option for those who remain refractory to oral formulations, or who cannot tolerate the side-effects. The intra vesicle administration of oxybutynin minimizes the anti cholinergic side-effects whilst retaining clinical efficacy.

Peterlangguth ,et al., (1997) have reported on the the Intestinal absorption of the quaternary trospium chloride : permeability –lowering Factor and bio-availabilities for oral dosage forms. the intestinal absorption mechanism, permeability and bioavailability of the para-sympatholytic, trospium chloride has been investigated *in vitro* and *in vivo* in rats , in order to

gain a better mechanistic explanation for the underlying cause leading to low bioavailability determinations were done in using-type chamber with rat Jejunum and human coca-2 cells. *In vivo* bioavailability and mass balance studied were done in rats. Absorption was studied from trospium chloride solutions in saline and from w/o micro emulsions and cyclo-dextrin complex formulation. The Absorption mechanism of trospium chloride across the intestinal epithelium is rather complex and involves p-glycoprotein – mediated secretion and saturable binding to intestinal mucus. Trospium permeability across the intestinal epithelium increases non linearly with rising drug concentration at the optical membrane, neither the micro-emulsion nor the cyclo-dextrin formulations increase the permeability of trospium *in vitro* , leading to lower or equal bioavailability of these *in vivo* as compared with the aqueous solution control

Donald m. et al., (1999) have reported on the evaluation of a new once-daily formulation of oxybutynin for the treatment of urinary urge incontinence and to evaluated in a 16-center, single-treatment study once-daily controlled-release oxybutynin (Ditropan XL) for urinary urge incontinence. Two hundred fifty-six participants with urge incontinence or mixed incontinence with a significant urge component were treated. After baseline measurements, participants converting from conventional oxybutynin started Ditropan XL at their previous oxybutynin dose; others started at 5 mg/day. Doses were adjusted until participants reached a maintenance dose that produced continence or the best balance between continence and side effects. This dose was continued for 12 weeks. Effectiveness was assessed by urinary diaries. Effectiveness was achieved across all doses studied, with 70% of participants using maintenance doses of 5 to 15 mg/day. Mean urge incontinence episodes per week decreased from 18.8 at baseline to 3.9 in maintenance week 1, 2.7 in week 4, and 2.8 at the end of the study. For those participants who reported urge incontinence episodes at baseline but were free of urge incontinence at maintenance week 1, 31% remained free of urge incontinence at every subsequent assessment. Participants who converted from other medications showed symptomatic improvement after conversion. At some time during the study, 58.6% of participants reported dry mouth, with 23.0% of participants rating it moderate or severe. Only 1.6% of participants discontinued the medication because of dry mouth. Ditropan XL treatment reduced the number of incontinence episodes. Maximum benefit was demonstrated by maintenance week 4 and was sustained through 12 weeks of maintenance therapy.

Darioush Dadgar et al., (1995) have reported the Issues in evaluation of bio-analytical method selectivity and drug stability four key issues were addressed: the statistical relevance of any selectivity test performed; a criterion for significant interference; experimental methods to establish selectivity; and criteria for acceptance. To ensure that compound integrity is maintained throughout the work-up process, statistically meaningful methods of stability evaluation which are associated with specific acceptance criteria are required. Suitable methods for evaluating stability of analyte and/or solutions of analyte, in process stability, processed sample stability, long term stability and freeze-thaw stability, as well as meaningful acceptance criteria, are presented

L. Cardozo et al., (2000) have reported on the Efficacy of trospium **c**hloride in patients with detrusor instability: a placebo-controlled, randomized, double-blind, multi-centre clinical trial the main objective to assess the efficacy and safety of trospium chloride in the treatment of detrusor instability, compared with placebo. Patients and methods In all, 208 patients were allocated at random to either trospium **c**hloride or placebo in a double-blind clinical study; the patients were treated for 3 weeks .Uro-dynamic values were measured[37] at the beginning and end of the treatment period. Adverse events were recorded on patient diary cards. a confirmatory adaptive procedure with one planned interim analysis was used to evaluate efficacy. Trospium chloride produced significant improvements in maximum cysto-metric bladder capacity and urinary volume at unstable contraction. The patients' assessment of efficacy showed significantly greater clinical improvement in the trospium **c**hloride group than in the placebo group Furthermore, trospium **c**hloride was well tolerated, with similar frequencies of adverse events reported in both groups . Trospium chloride (20mg twice daily) is an effective and safe option for the treatment of detrusor instability.

P. Lopez pereira et al., (2003) have investigated that trospium chloride for the treatment of detrusor instability in children, assessed the efficacy and most appropriate dosage of trospium

chloride for managing bladder instability in children as compared with a placebo. a total of 58 patients with bladder instability were allocated at random to 1 of 5 groups—10, 15, 20 or 25 mg trospium chloride, or placebo administered daily in a multi-center , randomized, single-blind clinical study. Patients were treated for 21 days, and current symptoms, voiding diary and uro-dynamic values were collected at the beginning and end of the treatment period. All adverse events were recorded at the last visit. 50 patients treated with trospium chloride 41 had a positive therapeutic result versus only 3 of 8 patients with improvement in the placebo group

CHAPTER-4

TROSPIUM CHLORIDE DRUG PROFILE

Trospium chloride is a quaternary ammonium compound which is used to treat overactive bladder and symptoms of urinary incontinence, frequency, and urgency. Trospium chloride is a fine, colorless to slightly yellow crystalline solid. The compounds solubility in water is approximately 1g/2ml.each capsule contains the following in active ingredients: sugar spheres, Metha acrylic acid copolymer, ethyl cellulose, hydroxyl propyl methyl cellulose, tri ethyl citrate.

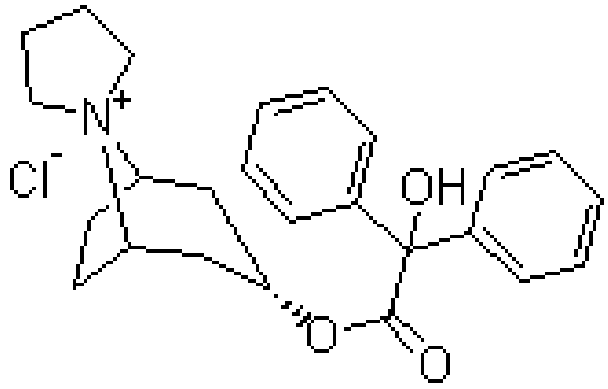


FIG: 4.1 MOLECULAR STRUCTURE OF TROSPIUM

MOLECULAR FORMULA : $C_{25}H_{30}NO_3$
MOLECULAR MASS : 427.9
CHEMICAL NAME : Spiro[8-azoniobycyclo[3,2,1]octane-8,1-pyrrolidinium]-3 -[(hydroxydiphenyl-acetyl)-oxy] Chloride(1α,3β,5α)-(9Cl)

PHARMACOLOGY:

Acetylcholine receptors:

The acetylcholine receptors are subdivided into Nicotinic and Muscarinic receptor subtypes. These receptors are also called as cholinoreceptor. The term cholinoreceptor means the receptors which respond to acetylcholine.

I) Nicotinic Receptors:

The receptors located on neuromuscular junction (NMJ) and at all autonomic ganglia (including adrenal medulla) are nicotinic receptors. The nicotinic receptor subtype are classified as muscle type (NM) and neuronal type (NN) and central nicotinic receptors.

II) Muscarinic Receptors:

The receptors present at the parasympathetic neuro-effector junction at all smooth muscles and glands are muscarinic cholinergic receptors. These receptors are currently divided into five sub-types(M_1,M_2,M_3,M_4,M_5). *In-vitro* receptor binding studies have demonstrated the selectivity of trospium chloride for muscarinic receptor over nicotinic receptors, and similar affinity for the M_2 and M_3 muscarinic receptor subtypes. M_2 and M_3 receptors are found on the glandular and visceral smooth muscles. Their activation results in mainly excitatory effects, e.g., increase in sweating, bronchial and salivary secretions, the contraction of visceral smooth muscles.M_1, M_4,M_5 receptors are largely confined to CNS but their functional role is poorly understood.

MECHANISM OF ACTION:

Trospium chloride is an antispasmodic, anti-muscarine agent.. Trospium works by blocking cholinergic receptors that are found on muscle cells in the wall of the bladder, hence it is said to have anti-cholinergic property. Normally a natural body chemical called acetylcholine acts on the cholinergic receptors under the body's control and this causes the bladder muscle to contract and the bladder to empty. Sometimes the bladder muscle can contract uncontrollably, causing the bladder to empty too frequently or

unexpectedly Trospium chloride compete with acetylcholine or other muscarinic agonists for the common binding site on the muscarinic receptor (M1 to M5). Hence this drug is said to be competitive agonists of the action of acetylcholine and other muscarinic agonists. This antagonism is reversible and therefore the blockade by smaller doses of trospium can be overcome by the larger concentration of acetylcholine or muscarinic agonists. When trospium

binds to muscarinic receptor it blocks all action that is brought about by acetylcholine or muscarinic agonists. As trospium blocks the cholinergic receptors on the bladder wall, it prevents the action of acetylcholine. This relaxes the bladder muscle and helps making the bladder more stable.

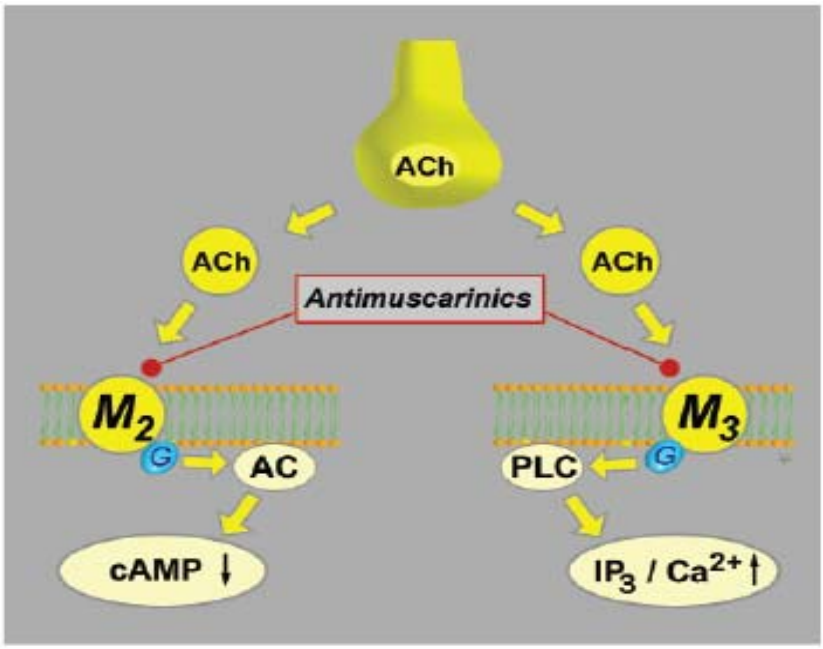


FIG: 4.2 MECHANISM OF ACTION OF ANTIMUSCARINIC DRUG TROSPIUM CHLORIDE

PHARMACOKINETICS:

Absorption: After oral administration of the drug (Trospium chloride) less than 10% of dose is absorbed. Mean absolute bioavailability of 20mg dose is 9.6%.Peak plasma concentration (C_{max}) occur between 5 to 6 hours post dose. A 3 fold to 4 fold increase in C_{max} was observed for dose increases from 20 mg to 40mg and from 20mg to 60mg respectively.

Distribution: Protein binding ranged from 48 to 78% depending upon the assessment method used, when a range of concentration levels of trospium chloride (0.5-100µg/L) were incubated in

vitro with human serum. The ratio of trospium chloride in plasma to whole blood was 1:6:1.This ratio indicates that the majority of trospium chloride is distributed in plasma.

Metabolism: The metabolic pathway of trospium chloride in humans has not been fully defined. Of the dose absorbed following oral administration, metabolites account for approximately 40% of the excreted dose. The major metabolic pathway of trospium is hypothesized as ester hydrolysis with subsequent conjugation of benzylic acid to form azoniospironortropanol with glucaronic acid.

Excretion: The plasma half life for trospium chloride following oral administration is approximately 35 hours. After oral administration of trospium chloride, a majority of dose was recovered in faeces (85.2%) and a smaller amount of dose (5.8%) in urine. Of the radioactivity excreted into the urine 60% was unchanged trospium.

DRUG INTERACTIONS:

Trospium is metabolized by ester hydrolysis and excreted by the kidneys through a combination of glomerular filtration .and tubular secretion. No clinically relevant metabolic drug-drug interactions are anticipated with trospium. However some drugs which are actively secreted by the kidney may interact with trospium by competing for renal tubular secretion.

The concomitant use of trospium with other antimuscarinic agents that produce dry mouth and constipation and other anticholinergic effects may increase the frequency or severity of such effects. Trospium may potentially alter the absorption of some concomitantly administered drugs due to anti-cholinergic effects of gastro intestinal motility.

Food interaction:

Administration of Trospium immediately after a high fat content meal reduced the oral bio availability of Trospium Chloride by 35% for AUC(0-Tlast) and by 60% for C_{max}. It is therefore recommended to take trospium on an empty stomach at least one hour before a meal.

Alcohol interaction:

Alcohol should not be consumed within two hours of Trospium administration. Alcohol may enhance the drowsiness caused by anti-cholinergic agents.

TOXICOLOGY:

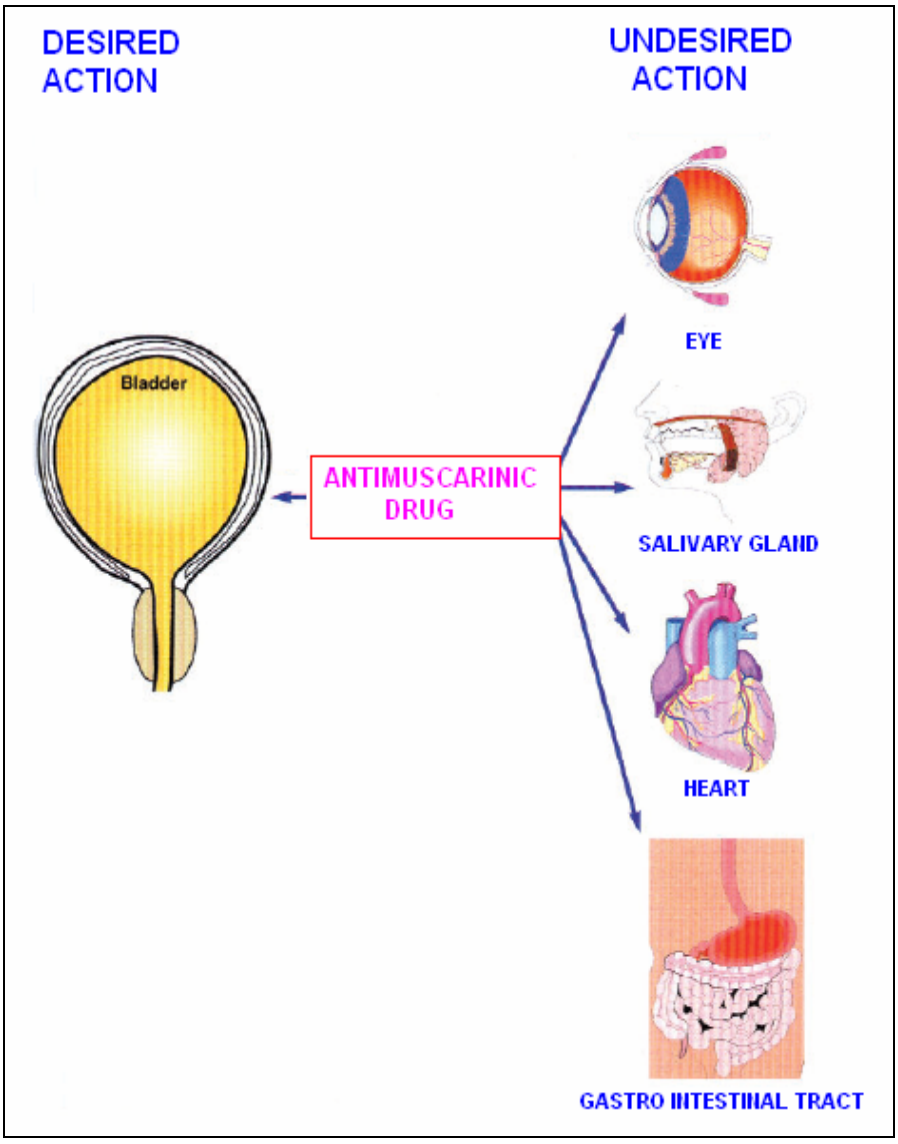
EFFECTS ON DIFFERENT ORGAN SYSTEM:

The effectiveness of trospium chloride varies according to the tissue.

The following sequence of sensitiveness of different smooth muscles and glands toward trospium action.

Sweat, Bronchial and Salivary glands >> Heart and Eye >> Bladder and Gastrointestinal tract>> Gastric glands.

FIG: 4.3 EFFECT OF TROSPIUM ON DIFFERENT ORGAN SYSTEM



Central Nervous System:

Trospium chloride as almost no detectable CNS effects in low doses. In therapeutic doses it has only mild stimulant effect on medulary centres in higher doses it stimulates higher cerebral centres and in toxic doses the central excitation becomes more prominent leading to restlessness, irritability, disorientation, hallucinations. With still larger doses, the stimulation is followed by depression leading to circulatory collapse, paralysis, coma and respiratory failure leading to death.

Eye:

Circular muscle of iris, ciliary muscle and lacrimal glands possess M_3 receptors. When instilled in eye, trospium or other tertiary ammine antimuscarinic drugs, block M_3 receptors present in pupillary constrictor muscle and produce mydriasis, which is a consequence of unopposed sympathetic dilator activity. Thus in true sense, this type of mydriasis is passive (indirect) mydriasis. As normal papillary responses being blocked, the eyes become unresponsive to light (lose of light reflects).

Cardio Vascular System:

With usual clinical doses trospium causes a transient bradycardia initially. The bradycardia is rarely significant(only 5 to 8 beats lesser per mininute than normal) and are usually absent after rapid injection. There are no changes in blood pressure or cardiac output. This bradycardia occurs due to blockade of M_1 receptor, which releases acetyl choline sinoarticular node

Higher dosage of trospium dilate cutanious blood vessels, especially of the face. The mechanism of this anomalous response is not known. However this would happen either due to histamine release or due to direct compensatory vasodialotor activity of trospium.

Respiratory System:

Both smooth muscle and secretory glands of the pulmonary airway receive vagal parasympathetic innervation and have predominantly M_3 receptors. Trospium like drugs inhibit secretion of nose, mouth, pharynx and bronchi and thus dry the mucous membrane of the respiratory tract. This drugs also reduce the laryngospasm during general anaesthesia. However, drying of mucus secretion and suppuration of muco-ciliary clearance are undesirable side effects of trospium in patients with airway disease as this leads to the formation of mucous plugs which can dangerously obstruct the airflow and predispose the patient to infection

Gastro Intestinal Tract:

I) Gastric secretions: Trospium can reduce the basal secretion of gastric acid by 40-50%. The concentration of the acid is not necessarily lowered as secretion of HCO_3^- is also blocked along with H^+. Not only the volume of secretion, but the total amount of H^+, HCO pepsin, and mucin, are all reduced. The effects of trospium on salivary secretion in more marked. The mouth becomes dry and tus swallowing as well as talking becomes difficult.

II) Motility: Trospium is very effective in reducing the tone and motility of the gut, right from stomach to colon. This results in prolongation of gastric emptying time, decrease in tone, amplitude and frequency of peristaltic movements.

Genitourinary Tract:

The smooth muscle of ureters and urinary bladder wall is relaxed by trospium and hence the voiding is slowed (urinary retention). The antimuscarinic drug have no significant effect on uterus, although these drugs cross placental barrier, the foetus is apparently not affected.

Sweat Glands:

Thermoregulatory sweating is suppressed by trospium. Sweat glands have M_3 receptors and are innervated by cholinergic nerves which are sympathetic in origin. In adults the skin may become dry and hot but the rise in body temperature results only in toxic doses.

CONTRAINDICATIONS:

Trospium is contraindicated in patients with Urinary retention, Gastric retention, or uncontrolled narrow-angle glaucoma and in patients who are at risk for these conditions. Trospium chloride is also contraindicated in patients who have demonstrated hypersensitivity to the drug or any of its ingredients.

OVERDOSAGE:

Anti-muscarinic drugs are widely used in the treatment of urinary incontinence. However these drugs lack selectivity for the bladder, and effects on other organ system may result in side effects. Over dosage with anti-muscarinic drugs including trospium chloride can result in severe anti-muscarinic effects.

Symptoms*:* A 7-month-old baby experienced tachycardia and mydriasis after administration of a single dose of 10 mg trospium given by a sibling. The baby's weight was reported as 5 kg. Following admission into the hospital and approximately 1 hour after ingestion of the trospium, medicinal charcoal was administered for detoxification. While hospitalized, the baby experienced mydriasis and tachycardia up to 230 beats/min. Therapeutic intervention was not deemed necessary. The baby was discharged as completely recovered the following day.

Treatment: Over dosage with trospium may result in severe anticholinergic effects. Treatment should be provided according to symptoms and supportive care. In the event of over dosage, electrocardiogram monitoring is recommended.

PHARMACOLOGY IN SPECIAL POPULATION:

Special populations –*Pharmacokinetics*

Gender: Studies comparing the pharmacokinetics in different genders had conflicting results. When a single 40 mg trospium dose was administered to 16 elderly subjects, exposure was 45% lower in elderly females compared with elderly males. When 20 mg trospium was dosed twice daily for 4 days to 6 elderly males and 6 elderly females (60 to 75 years of age), AUC and C_{max} were 26% and 68% higher, respectively, in females without hormone replacement therapy than in males.

Renal function impairment: Severe renal impairment significantly altered the disposition of trospium. A 4.5-fold and 2-fold increase in mean AUC and C_{max}, respectively, and the appearance of an additional elimination phase with a long half-life (approximately 33 hours) was detected in patients with severe renal insufficiency compared with healthy, nearly age-matched subjects. The different pharmacokinetic behavior of trospium in patients with severe renal insufficiency necessitates adjustment of dosage frequency. The pharmacokinetics of trospium have not been studied in people with mild or moderate renal impairment from 30 to 80 mL/min

Hepatic function impairment*:* C_{max} increased 12% and 63% in subjects with mild and moderate hepatic impairment, respectively, compared with healthy subjects. AUC was similar. Trospium must be used with caution in hepatic impairment dysfunction.

Special populations-*Safety and efficacy:*

Elderly: In 2 studies, the incidence of commonly reported anti-cholinergic adverse events in patients treated with trospium (including dry mouth, constipation, dyspepsia, UTI, and urinary retention) was higher in patients 75 years of age and older as compared with younger patients. This effect may be related to an enhanced sensitivity to anti-cholinergic agents in this patient population

Pregnancy*:*. Trospium has been shown to cause maternal toxicity in rats and a decrease in fetal survival in rats administered approximately 10 times the expected clinical exposure (AUC). There are no adequate and well-controlled studies in pregnant women. Use during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Lactation: Trospium (2 mg/kg orally and 50 mcg/kg IV) was excreted, to a limited extent (less than 1%), into the milk of lactating rats. The activity observed in the milk was primarily from the parent compound. It is not known whether this drug is excreted in human milk. Exercise caution when administering trospium to a nursing mother. Use during lactation only if the potential benefit justifies the potential risk to the newborn.

Children: Safety and efficacy in pediatric patients have not been established.

CHAPTER -5

METHODOLOGY

STUDY SITE

The study was conducted at WELLQUEST CLINICAL RESEARCH a division of PIRAMAL HAELTH CARE LIMITED.

COMPANY PROFILE

Wellquest clinical research labs have a 52 bedded facility, located in Ramanthapur HYDERABAD. This unit conducts Bioavailability and Bioequivalence clinical studies on healthy human volunteers. The unit has been organized into various sections such as: Counseling Area, Examination Room, Fully Equipped I.C.U.(Emergency Room), Blood Sampling Area, Volunteer Housing Area, Dining Area, Recreation Area and Documentation Room.

5.1 CONTRACT BETWEEN SPONSOR AND INVESTIGATOR

At first, the sponsor takes responsibility for the initiation, management, and/or financing of a clinical study of a drug and signs a contract with a **Contract Research Organization (CRO)**, for initiating and conducting the study. The investigator agrees and signs the protocol with the sponsor, and confirms in writing that he reads, understands and will work according to the protocol and Good Clinical Practice. The investigator is responsible for ensuring that the protocol is strictly followed.

5.2 PROTOCOL PREPARATION

After signing the agreement, the investigator starts preparing a protocol for the specific drug product which gives the background and rationale for the study. The contents of a protocol generally include the below mentioned topics:

A. GENERAL INFORMATION

- Protocol title, protocol identifying number, and date. Any amendment(s) also bear the amendment number(s) and date(s).
- Name and address of the sponsor and monitor (if other than the sponsor).
- Name and title of the person(s) authorized to sign the protocol and the protocol amendment(s) for the sponsor.
- Name and title of the investigator(s) who is (are) responsible for conducting the study, and the address and telephone number(s) of the trial site(s).
- Name(s) and address of the clinical laboratory and other medical and/or technical department(s) and/or institutions involved in the study.

B. BACKGROUND INFORMATION

- Name and description of the investigational product.
- A summary of findings from non clinical studies that potentially have clinical significance and from clinical trials that is relevant to the study.
- Summary of the known and potential risks and benefits, if any, to human subjects.
- Description of the population to be studied.
- References to literature and data that are relevant to the trial and that provide background for the trial.

C. STUDY DESIGN

The scientific integrity of the study and the credibility of the data from the study depend substantially on the study design. A description of the trial design should include:

- A description of the measured taken to minimize/avoid bias, including Randomization.
- A description of the study treatment(s) and the dosage and dosage regimen of the investigational product. Also include a description of the dosage form, packaging, and labeling of the investigational product.
- The expected duration of subject participation, and a description of the sequence and duration of all study periods, including follow-up, if any.
- A description of the "stopping rules" or "discontinuation criteria" for individual subjects, parts of study and entire study.
- Accountability procedures for the investigational product.

D. SELECTION AND WITHDRAWAL OF SUBJECTS

- Subjects inclusion criteria.
- Subject exclusion criteria.
- Subject withdrawal criteria.

E. TREATMENT OF SUBJECTS

- The treatment to be administered, including the name of the product, the dose, the dosing schedule, the route/mode of administration, and the treatment period(s), including the follow-up period(s) for subjects for each investigational product treatment/study treatment group/arm of the study.
- Medication/treatment permitted (including rescue medication) and not permitted before and/or during the study.
- Procedures for monitoring subject compliance.

F. DIRECT ACCESS TO SOURCE DATA/DOCUMENTS

The sponsor ensures that it is specified in the protocol or other written agreement that the investigator(s)/institution will permit study –related monitoring, audits, IRB/IEC review, and regulatory inspection(s), providing direct access to source data/document(s).

G. INDEPENDENT ETHICS COMMITTEE (IEC) APPROVAL

After the protocol preparation for the particular study, Independent Ethics Committee approval is taken for conducting the study, whose responsibility is to ensure the protection of the rights, safety and well-being of human subjects involved in a trial and to provide public assurance of that protection, by, among other things, reviewing and approving/providing favorable opinion on, the trial protocol, the suitability of the investigator(s), facilities, and the methods and material to be used in obtaining and documenting informed consent of the trial subjects. The legal status, composition, function, operations and regulatory requirements pertaining to Independent Ethics Committees may differ among countries. After the IEC approval, the study process commences.

5.3 STUDY CRITERIA RECRUITMENT AND SCREENING OF HEALTHY VOLUNTEERS

Volunteers were screened through a screening procedure within 29 days prior to the first dose administration or as specified by the protocol. The following medical tests were done during the screening process:

- ECG
- X-Ray
- Laboratory tests (serology, hematology, urine analysis and biochemistry)

INCLUSION CRITERIA

- Healthy males aged between 18 and 45 years. Weighing at least 50kg and a body mass index of 18.5 kg/m^2 and 24.99 kg/m^2.
- Completed the screening process prior to the study.
- Subjects who have no evidence of underlying disease during screening medical history, physical examination, laboratory evaluation, ECG, X-ray recordings.
- Willingness to provide written informed consent to participate in the study.
- Availability of volunteer to the entire study period.
- Willing to take non-vegetarian diet.

EXCLUSION CRITERIA

- History or presence of significant cardiovascular, pulmonary, hepatic, renal, hematological, gastro – intestinal, endocrine, immunologic, dermatologic, neurological or psychiatric disease.
- History of smoking more than 5 cigarettes per day or consumption of other forms of tobacco containing products.
- History of alcohol intake more than 3 units/day.
- History of drug allergy to the test drug or any drug chemically similar to the drug under investigation.
- Volunteers who have donated more than 450ml of blood in the past 3 months.
- Subjects who have participated in another clinical study in the past 12 weeks of commencement of the study.

WITHDRAWAL CRITERIA

- Adverse event which warrants withdrawal of subject.
- Concurrent medication.
- Erroneous inclusion in study.
- Failure to comply with restrictions and prohibitions.
- Failure of drug administration.
- Inadequate cooperation.

Any subject withdrawal during the study shall be handled as per in-house procedure. The date on which the subject is withdrawn from the study and the reason for discontinuation will be recorded.

RESTRICTIONS

Before entering into the study subjects have to follow below mentioned restrictions.

- No subject should receive any medications [including OTC products], for the 7 days preceding the study.
- Smoking should be prohibited from 24 hours before check in and until the subject leaves the facility.

- The use of alcohol containing beverages should be prohibited for 48 hours before the check in process for all the periods and throughout the study period.
- The use of caffeine/xanthine containing beverages and foods should be prohibited for 24 hours before check in for all the periods and throughout the period of sample collection.
- No grapefruit containing foods and juices should be permitted for 72 hours prior to check in all the periods.

5.4 STUDY PROCEDURE

The volunteers who met the inclusion and exclusion criteria were allowed to enter the study. 20 volunteers were allowed to participate in the study as per the protocol requirement.

The study is of two periods and each period involves three stages:

1. Check-in
2. Dosing
3. Check-out

5.5 PERIOD-ONE

5.5.1 CHECK-IN PROCEDURE

As soon as the volunteer reaches the clinical centre he was registered into the volunteer entry logbook and then will be counseled in-groups by the study counselor regarding:

- Introduction of the research centre,
- Brief explanation of Clinical Bio-studies (Bioavailability and Bioequivalence),
- The purpose of such Clinical Bio studies,
- Study procedure,
- Number of blood draws,
- Ambulatory blood draws,
- Probable date of Check-in and Check-out,
- Duration of stay in MTR,
- Risks and benefits for volunteers,

- Compensation to volunteers,
- Volunteer rights,
- Safety and confidentiality aspects,
- Procedure for informed consent, and other details related to the particular study.

  Sufficient time is allowed after counseling for the volunteer to express his willingness to participate in the study. He was also informed that he is free to withdraw from the study any time they want.

5.5.2 SIGNING INFORMED CONSENT

Then an oral presentation of Informed Consent was done. Informed consent form is a process by which a subject voluntarily confirms his willingness to participate in a particular study, after having been informed of all aspects of the study that are relevant to the subject's decision to participate. Informed consent is documented by means of a written, signed and dated informed consent form.

Volunteers are also given opportunities to ask their doubts and to make a decision. Copy of ICF is issued to the subjects and sufficient time is allotted to read and understand the content of ICF.

The content of ICF includes study title, purpose and background of the study, study procedure, number of blood samples to be collected, restrictions to be followed before and after the study, adverse reaction of the drug, risks or discomforts associated with participation in the study, compensation, medical treatment for injury and rules and regulations to be followed. All the above points are discussed in detail in ICF. The healthy volunteers willing to participate in the study have to sign the ICF. They were given token numbers according to their arrival time to the centre and the token number is considered as the volunteer's subject number.

5.5.3 ELIGIBILITY ASSESSMENT

After their consent, the subject's vitals (BP, pulse rate, temperature and respiratory rate) are checked by duty doctor. If subject's vitals are normal and if the subject has followed all the restriction mentioned above, then the subject is eligible to participate in the study.

5.5.4 VOLUNTEER HOUSING

The subjects are provided with separate lockers to keep their belongings and also a dress to change, along with a pair of slippers and other toiletries. After changing, the subjects are

allowed to enter the housing area where the custodian discusses regarding Do's and Don'ts with the subjects. Then subjects are provided with identity cards bearing their photos and other identification details like, subjects are provided with balanced standardized meal, which is planned by a dietician.

5.5.5 DISPENSING OF DRUG

The study drugs are stored according to the storage condition mentioned in the protocol. Temperature and humidity of the storage area is recorded at regular interval. The pharmacist dispenses the medicines a day before dosing in presence of quality assurance personnel. The dispersing of drug is done according to the randomization schedule and is pre-labeled (which includes subject number and study number) unit dose plastic container with lid and then the plastic containers are placed in a pre-labeled paper envelope. Drugs present in the plastic container are used during dosing process.

5.5.6 DOSING

On the dosing day, subjects are given a wakeup call by the custodian at a specific time and they are also given time to freshen up, after which they are called number wise for cannulation. A pre-dose sample was collected 1 hour prior to dosing which was called zero hour samples. Dosing was done in the morning, at the time mentioned in the protocol. The personnel designated and the clinical investigator was responsible for the dosing activity. Subjects are called in batches at pre assigned stations where the subjects receive single oral dose of (standard drug or test drug as per the randomization schedule) assigned formulation with 240±2ml of water. The volunteers were administered with the drug product after the over night fasting, since the study is under fast conditions. Single tablet of Trospium chloride was administered in an empty stomach orally to the subject with 240±2 ml of water, in sitting posture at ambient temperature in the morning

5.5.7 RANDOMIZATION

The order of receiving the test and reference products for each subject during all the periods of the study will be determined according to a randomization schedule. The randomization schedule will be generated with the SAS software; version 9.1 package (SAS Institute Inc., USA).The randomization schedule will be kept under controlled access.

SUBJECT NO	SEQUENCE	PERIOD 1	PERIOD 2
S01	2	R	T
S02	1	T	R
S03	1	T	R
S04	2	R	T
S05	2	R	T
S06	1	T	R

TABLE:5.1 RANDOMIZATION SHEDULE

S07	2	R	T
S08	1	T	R
S09	1	T	R
S10	2	R	T
S11	2	R	T
S12	1	T	R
S13	1	T	R
S14	2	R	T

R - Reference; T – Test

5.5.8 RESTRICTIONS:

The following restrictions were maintained in order to study and evaluate the absorption level of trospium chloride in plasma.

- Pre dose fasting -at least 10 hours
- Post dose fasting - 04 hours
- Water restriction -Pre dose and Post dose 01hour
- Posture restriction -04 hours post dose in sitting.
- Caffeine such as coffee, tea, chocolate, and all caffeine containing soft drinks or energy drinks, alcoholic beverages, grapefruit was restricted for 48 hours prior to initial dosing and throughout the study until last scheduled blood sample collection.

5.5.9 FOOD ADMINISTRATION:

- Subjects were given lunch of 1100 kcal after 04 hours of dosing, snacks of 500 kcal at 08 hours post dose and dinner of 900 kc12 hours post-dose.

5.5.10 BLOOD SAMPLING AND HANDLING:

- A total of 22 blood samples were collected during each period through an indwelling intravenous cannulation placed in a fore arm vein.
- The pre-dose samples were collected within 90 minutes before dosing and post- dose sample were collected within 2 minutes of the scheduled time.
- The blood samples were collected using syringe and adaptor and transferred into pre-

labeled K_2 EDTA vaccutainers placed upright in a rack kept in ice bath until centrifugation.

- Intravenous indwelling cannula was kept *in situ* as long as possible by injecting 0.5ml of 10 IU/ml of heparin in normal saline solution to maintain the cannula patent.
- Blood samples were collected after discarding the first 0.5ml of heparinized blood from tubing.
- The cannula was withdrawn after the collection of 24.00 hours sample or earlier if blocked.
- The samples were withdrawn at the following time points

TABLE: 5.2 BLOOD SAMPLING TIME POINTS

S NO	TIME POINTS(Hours)
1	1.00
2	2.00
3	3.00
4	4.00
5	4.33
6	4.67
7	5.00
8	5.33
9	5.67
10	6.00
11	6.33
12	6.67
13	7.00
14	8.00
15	9.00
16	10.00
17	12.00
18	24.00
19	48.00

20	72.00
21	96.00

- The blood loss per subject for the entire study not exceeded 253ml.The split up is as follows.

  010ml -Screening prior to study

  010ml -Pre-dose blood draw (05 ml for each period)

  210ml -Blood draws (21 draws×2periods×05ml)

  018ml -Discarded blood (18 draws×2 periods×0.5 ml)

  010ml -Post dose investigations (05ml blood for each period)

5.5.11 SUBJECT MONITORING

Subjects were monitored throughout the study period for safety and adverse events. The following safety assessments were carried out to ensure the safety of Subjects.

- Clinical examination and vitals – Before check in, before check out of each Period, during ambulatory visit and at any other time if necessary.
- All post – dose vital sign measurements were recorded within ± 30 minutes of the scheduled time.
- Subjects were questioned for well being at the time of recording of vital signs and at any other time if necessary.
- Monitoring for adverse events - throughout the study period.

5.5.12 SEPARATION AND STORAGE OF BLOOD SAMPLES

- The blood samples were collected at regular time intervals. The vaccutainers containing the blood samples are centrifuged at 4000±50 rpm for 10 minutes at 4ºC as early as possible to separate plasma.
- In case of inadequate plasma, the samples was re-centrifuged
- The supernatant matrix (plasma) was transferred into pre-labeled poly propylene tubes (RIA Vials).
- Segregation was done as quickly as possible, as thawing may affect the quality of the sample adversely.

- Separate racks for each subject were kept in trays containing ice pieces to accommodate the samples.
- Samples were segregated according to the time point and subject numbers in corresponding racks.
- Segregated samples were placed in pre-labeled zip lock covers and were stored in Ultra Low Temperature Freezer at -70ºC.

5.5.13 CHECK-OUT

On the next day of dosing, after ensuring that all the activities related to the study are completed, the cannula is removed. After the last blood draw and the investigator checked the vitals of the subjects. The check-out procedure was followed only if the health status of the subject is normal and here the subject is informed to contact the investigator in case of any medical problems related to the study. After this, subjects were given the locker keys and asked to change to their own clothes. They were checked out after partial payment was hanged over to the subjects at the end of the first period. The subjects are informed about the date of period – so that they can reach the centre at appropriate time for the second period.

5.5.14 WASHOUT PERIOD

Subsequent treatment is separated by periods long enough in order to eliminate the previous dose before the next one. The two phases of treatment are separated by an adequate washout period of 10 days which is ideally equal to or more than five half life's of the moieties to be measured.

5.6 PERIOD – TWO

After the specified washout period, the second period of the study begins, where the subjects again return to the centre at predetermined date and time. Apart from the informed consent process, the remaining same procedure is carried out, such that the two periods are identical in every manner. In period – two, the subjects those who had received test drug during period one will receive standard drug and the subjects who had received standard drug during period one will receive test drug. During this period Subject No: 11 didn't come and he is considered as

withdrawn from the study and it is documented. At the end of the second period the subjects are provided with the full compensation

5.7 ANALYTICAL PROCEDURE

The Trospium Chloride test and reference product was compared using a validated Liquid Chromatography Mass Spectrometry method and according to the Bio-analytical laboratory's standard operating procedures and other applicable guidelines.

5.8 CHEMICALS USED

- Acetone M (HPLC Grade).
- Ammonium Formate (Analytical Grade).
- Trospium chloride (Working standard).
- Escitalopram oxalate (Internal standard)
- Formic acid (Analytical Grade).
- Acetone-M (HPLC Grade)
- Milli-Q Water (HPLC Grade).
- Ammonium acetate (GR Grade)

5.9 INSTRUMENT/EQUIPMENT USED

- LC-MS/MS
- HPLC
- Deep Freezer
- Vortex Shaker
- Ultrasonic Bath
- LV- Evaporator

5.10 Sample extraction procedure for Trospium Chloride

- Plasma samples were removed from the ultra low temperature freezer before 30 minutes of analysis and thawed to room temperature.
- 1ml of the sample was pipetted out in RIA vials and vortexed for 2 minutes.
- 50µl of International standard (IS) Esatilopram was added and then 400µl of Acetate in water was added and vortexed for 2 minutes.

Solid Phase Extraction:

- Solid phase extraction was used to isolate analyte from the solution. 30mg/ml catridges was used for solid phase extraction

Conditioning:

- 100µl of Acetone-M was added followed by 100µl of milli-Q water was added to condition the plexus 30mg/ml catridges. Conditioning was done to clean up the catridges before using the plasma samples.

Sample elution:

- 1ml of the sample was then loaded into the respective catridges and allowed to elute by maintaining positive pressure under vaccum.

Washing:

- The catridges were washed with 1ml of 5% ammonia in water and 1 ml of 1% formic acid in water.

- Selective washing was done to remove the impurities that were retained on the packing.

- The catridges were then allowed to dry well and samples were eluted using 0.5ml of 1% formic acid in Acetone-M.

- The eluted sample was vortexed and transferred into a clean glass tube

Positive pressure:

Positive pressure is a pressure within a system that is greater than the environment that surrounds the system.

- The sample was then evaporated to dryness under nitrogen using LV evaporator for 15 minutes at 40ºC and 15 psi pressure

- The samples were then reconstituted by adding 0.4ml of mobile Phase.

- The samples was then added into the glass vials and were Chromato- graphed in to the LC-MS/MS system along with quality control samples.

Table 5.3- Spiked Quality Control Samples of Trospium Chloride:

Stock QC ID	Stock Concentration (ng /ml)	Stock Aliquot (ml)	Diluent Added (ml)	Final Volume (ml)	Final Concentration (ng/ml)	Spiked CCID
QC 1	320.0000	0.2	9.8	10	6.4000	HQC
QC 2	208.0000	0.2	9.8	10	4.1600	MQC
QC 3	7.6960	0.2	9.8	10	0.1539	LQC
QC 4	2.7321	0.2	9.8	10	0.0546	LLOQ

5.11 CHROMATOGRAPHIC CONDITIONS

HPLC conditions

A summary of the chromatographic conditions is as follows:

Column : Thermo, Hypersil GOLD, 150X4.6mm. 5u

Mobile Phase	:	Acetone-M, 2mM Ammonium Formate (60:40)
Injection Volume	:	8 µl.
Flow Rate	:	0.8 ml/min
Column Oven temp	:	40°C
Auto sampler Temperature	:	4°C
Total Run Time	:	3.5 minutes

LCMS/MS conditions:

Molecule	**:**	Trospium(analyte)	scitalopram(Internal Standard)
Q_1 Mass (amu)	**:**	392.30	325.20
Q_3 Mass (amu)	**:**	**1**82.10	262.10
DP (Voltage)	**:**	95.00	95.00
EP (Voltage)	**:**	10.00	10.00
CE (voltage)	**:**	42.00	26.00
CXP (voltage)	**:**	16.00	16.00

Source Conditions :

GS1	**:**	40
GS2	**:**	55

Cur Gas **:** 30

Data processing:

Acquire chromatograms using the computer based analyst software supplied by Lab India Process data by peak area ratio. The concentration of the unknown is calculated from the following equation using regression analysis of spiked plasma calibration standard with the reciprocate of the drug concentration as a weighing factor (1 / concentration ^{2}):

$Y = mx + b$

Where, x = Concentration of Trospium

m = slope of the calibration curve

y = Peak area ratio

b = y – axis intercept of the calibration curve.

5.12 PHARMACOKINETIC ANALYSIS

The pharmacokinetic parameters C_{max}, AUC_{tot}, $AUC_{last,}$ T_{max}, $T_{½}$ of Trospium chloride were determined for test and reference product in each subject using kinetica^{tm} software (version 4.4.1)

Importing Files into Kinetica^{tm}

- Primary concentration data from Excel sheet was imported into Kinetica Software using Import Assistant Wizard.
- The Import Assistant Wizard is a series of dialogs that simplifies the importing of data and gives guidance through the step-by-step process.
- All datasets in the Excel file contain the same time column but different concentration column for each subject.
- XYi Data layout import method was selected and the source column called X and Y was inserted into the layout.

- The Import Assistant terminates once the process was complete and the imported data was displayed in Kinetica.
- The subject number (e.g. S01, S02……..), period (e.g. I, II…….), formulation (e.g. R or T) and the corresponding plasma concentration were entered in the appropriate dataset columns as per the Randomization schedule.
- All Missing or Lost samples resulting from clinical operations or sample separation was denoted by 'M' wherever applicable.
- The Missing samples were excluded from calculation of mean values.
- Concentration below the Lower Limit of Quantification (LOQ) was reported as Below Limit of Quantification (BLQ) and was set to Zero for all Pharmacokinetic Analysis.
- The pre-dose value less than 5% of C_{max} and more than LOQ was considered for Pharmacokinetic Analysis.
- The values more than 5% of C_{max} were not included for Pharmacokinetic Analysis.

AUC*

As a general practice, if the blood collection made beyond two mins of specified time, Pharmacokinetic Analysis were performed with the actual observed deviated time point. Such sampling time were tagged with an asterick(*) superscript wherever in the report table.

AUC Steady-state

All blood collection made within two mins of the specified time interval do not require any time correction for Pharmacokinetic Analysis. In such state AUC steady-state was selected.

Pharmacokinetic Analysis

The pharmacokinetic parameters were calculated and displayed in the data set view.

- X and Y axis, Mean curve was selected from the chart wizard
- The Appropriate concentration and time points were set to obtain the graph.
- Linear and semi-log plots of mean plasma concentration Vs time were obtained

CHAPTER-6

RESULTS AND DISCUSSION

6.1 LC-MS/MS ANAIYSIS OF TROSPIUM CHLORIDE

The plasma concentrations of Trospium chloride were analyzed using a validated Liquid Chromatography Mass Spectrometry (LC-MS/MS) method.

The concentrations of Trospium chloride present in 14 Healthy volunteers are summarized in the Table no: 6.1

6.2 PHARMACOKINETIC STUDY

Import individual concentration corresponding to the time points with the dosing regimen data from Excel sheet to the KINETICA^{tm} . The following pharmacokinetic parameters were obtained.

1. C_{max}
2. T_{max}
3. AUC_{last}
4. AUC_{tot}
5. K_{el}
6. $T_{1/2}$

Pharmacokinetic parameters obtained were listed in the Table no: 6.2

Fig :6.1 Representative regression analysis of a calibration curve for trospium chloride

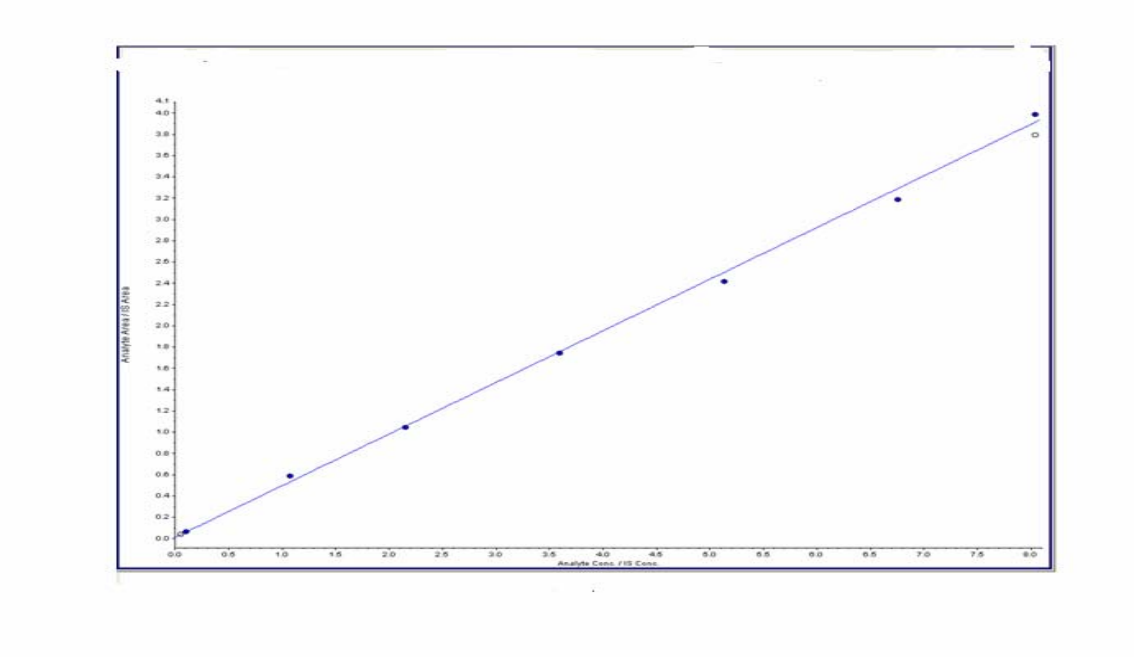


Table no 6.1 Individual concentration data of trospium chloride units:(ng/mL)

TIME (in Hrs)	S01 PI	S02PI	S03PI	S04PI	S05PI	S06PI	S07PI
0.00	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1.00	0.5770	2.3449	1.3326	2.0901	0.1785	2.4294	0.9969
2.00	0.8890	3.1048	2.8566	5.0338	0.4461	3.3031	2.9081

TIME (in	S01 PII	S02PII	S03PII	S04PII	S05PII	S06PII	S07PII
3.00	1.0211	3.5428	3.4381	8.6952	0.7557	2.5107	3.7501
4.00	1.1714	3.8791	2.9129	NS	0.8012	2.0422	4.3342
4.33	1.2760	4.8860	3.5873	12.0420	1.6302	2.2204	5.0459
4.67	1.4271	4.3743	3.5193	9.8667	1.5787	5.2846	1.8550
5.00	1.6064	4.0526	2.8575	8.9442	1.3316	1.6895	5.2333
5.33	1.1216	2.6114	2.7052	10.6689	1.1983	1.4825	5.5575
5.67	0.9614	3.6396	2.6684	8.9724	1.2832	1.3191	5.8779
6.00	1.8201	3.4120	2.3946	8.9619	1.2518	1.1119	5.0066
6.33	1.6608	3.0726	1.7461	2.9817	1.3081	0.9826	4.7752
6.67	1.7244	3.6615	1.8135	NS	1.2189	1.0671	3.7142
7.00	1.6413	2.8073	1.7846	6.9555	1.3062	0.9465	4.6555
8.00	1.3617	1.9963	1.3418	6.2541	0.9310	0.7520	4.3564
9.00	2.0863	1.3509	1.2425	4.9214	0.8237	0.6717	3.4011
10.00	1.7267	0.9710	1.0076	4.9637	0.6694	0.6350	2.5519
12.00	1.3838	0.7048	0.7958	3.5451	0.3915	0.5627	1.6970
24.00	0.7006	0.4712	0.5212	1.5585	0.3839	0.3658	0.6536
48.00	0.2648	0.2498	0.1501	0.4486	0.1274	0.1133	0.1473
72.00	0.0926	0.0791	BLQ	0.2078	0.0513	BLQ	BLQ
96.00	BLQ	BLQ	BLQ	0.1115	BLQ	BLQ	BLQ

(Cont ….)

Individual concentration data of trospium chloride units: (ng/mL)

Hrs)							
0.00	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1.00	0.5040	2.8967	0.3215	5.5960	0.1831	1.5350	0.9473
2.00	0.3676	7.2057	0.5966	7.7037	0.3641	1.7713	1.9554
3.00	0.4980	8.1366	0.6150	10.1613	0.7873	1.1843	3.1579
4.00	0.5222	7.0044	0.5762	10.9047	1.0772	0.8533	3.2751
4.33	0.8338	6.2226	0.8270	7.9557	1.8683	0.9258	4.3993
4.67	0.6512	NS	0.7398	10.2039	1.6630	0.9250	4.8790
5.00	0.9243	5.4882	0.6286	9.0009	1.4980	0.7395	4.7793
5.33	0.9023	6.6018	0.5001	9.4218	1.2446	0.6381	4.1246
5.67	0.8483	6.2384	0.5988	6.8259	1.2827	0.5869	4.4381
6.00	0.8461	5.9172	0.4573	7.7172	1.2979	0.4909	3.6687
6.33	0.8327	5.4714	0.4166	8.6784	1.3376	0.4576	3.7195
6.67	0.7829	5.5634	0.3680	7.5386	1.4393	0.4670	4.6547
7.00	0.7041	4.8595	0.4024	6.7966	1.3912	0.4092	3.5500
8.00	0.6078	3.7379	0.3017	6.4568	1.2616	0.3816	3.2437
9.00	0.5905	3.5561	0.2706	5.7658	0.9830	0.3541	2.6648
10.00	0.4810	3.0126	0.1733	4.8553	0.7322	0.3033	1.9238
12.00	0.3560	1.9234	0.2197	3.5779	0.6496	0.2564	1.3789
24.00	0.2155	0.9215	0.1583	1.4892	0.3770	0.1899	0.4303
48.00	0.1187	0.2534	BLQ	M	0.1225	0.0803	0.0957
72.00	0.0922	BLQ	BLQ	0.1836	BLQ	BLQ	BLQ
96.00	0.0596	BLQ	BLQ	0.0987	BLQ	BLQ	BLQ

TIME (in Hrs)	S08PI	S09PI	S10PI	S11PI	S12PI	S13PI	S14PI
0.00	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1.00	2.1076	0.1428	1.2060	1.1984	0.8414	0.7169	1.9011
2.00	5.3097	1.0165	2.2498	1.2626	0.8342	1.8943	9.7236
3.00	12.6009	1.8177	4.9956	1.4704	0.8514	2.6023	9.9273

4.00	7.7034	2.2651	7.1838	1.6227	0.9437	2.5890	9.3045
4.33	14.3223	2.9133	10.1514	2.5151	1.4680	2.8641	12.6207
4.67	13.6608	2.6122	11.8206	2.5976	1.6642	2.8311	11.7456
5.00	11.1981	2.6449	9.4824	2.2752	1.4906	2.7395	9.3378
5.33	10.8375	2.4109	8.8164	2.1127	1.3432	2.7176	8.0796
5.67	7.9047	1.9542	10.1586	2.0667	1.1705	2.8477	7.8264
6.00	8.8017	2.0662	8.2356	2.0750	1.2206	2.7855	6.9672
6.33	7.7811	1.9375	7.1088	1.4361	1.2819	2.5893	7.4470
6.67	6.8205	1.7206	7.4432	1.3365	1.1126	2.1379	4.9594
7.00	6.6542	1.7416	7.0071	1.3577	1.0106	2.1542	5.6202
8.00	5.6260	1.4244	6.7944	1.6330	0.8827	1.9877	5.9195
9.00	5.2937	1.2385	5.9553	1.2597	0.7486	1.6476	5.3906
10.00	3.9583	0.9998	4.8492	1.1563	0.5880	1.4603	4.9202
12.00	2.9114	0.7813	3.2632	0.9020	0.3796	1.2858	3.4366
24.00	1.1714	0.3753	1.4845	0.4994	0.2688	0.5581	1.9301
48.00	0.3527	0.1321	0.4524	0.1755	0.0931	0.1328	0.5733
72.00	0.1289	BLQ	0.1935	0.1157	0.0641	0.0641	0.2051
96.00	0.0589	BLQ	1.6132	BLQ	BLQ	BLQ	0.1071

(Cont….)

Individual concentration data of trospium chloride units:(ng/mL)

TIME (in Hrs)	S08PII	S09PII	S10PII	S11PII	S12PII	S13PII	S14PII
0.00	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
1.00	2.2375	0.2443	1.1960	0.6785	1.1815	0.4457	1.9003
2.00	6.6993	0.9263	2.6488	1.4855	3.5995	0.5072	3.2659
3.00	15.2682	2.3882	3.3098	2.0129	6.4744	0.5005	4.3511
4.00	17.5230	5.1353	4.4937	2.5174	7.4998	0.4579	4.5258
4.33	18.4716	6.8579	6.5431	3.6917	7.5354	0.5501	5.5326
4.67	17.0349	6.6503	7.1651	3.5604	6.5028	0.5350	5.9529
5.00	15.1839	6.4236	7.2021	2.9324	6.7126	0.5416	6.2613
5.33	14.6109	5.9679	7.5661	2.9289	6.5573	0.4246	5.4292

5.67	10.3215	4.5806	6.9466	3.0346	6.3512	0.3660	4.7900
6.00	11.7495	4.8630	6.9895	3.1923	5.5900	0.3818	4.3627
6.33	11.3001	4.6420	6.9291	3.3372	5.5445	0.3258	4.4526
6.67	12.1164	4.3023	7.1009	3.5871	4.9037	0.3098	3.3846
7.00	8.0967	4.1599	5.2683	3.3407	4.7043	0.3061	3.0967
8.00	3.3477	NS	4.6891	3.3250	3.6513	0.2915	2.8013
9.00	6.3480	3.2279	4.3297	2.5005	3.1404	0.2310	2.4803
10.00	4.5070	2.4701	3.3625	2.1383	2.1975	0.2184	2.5000
12.00	3.0476	1.8285	2.7307	1.9531	1.7582	0.1634	1.9336
24.00	1.2981	0.7476	1.2232	0.8844	0.5478	0.1343	1.0231
48.00	0.3273	0.2000	0.4618	0.3392	0.1348	0.0656	0.2970
72.00	0.1099	BLQ	0.1952	0.1551	M	BLQ	0.1522
96.00	BLQ	BLQ	0.0774	0.0700	BLQ	BLQ	0.0769

BLQ : Below Limit of Quantification (0.0513 ng/mL) NS : Insufficient Sample PI : Period One PII: Period two

Table no 6.2 Trospium pharmacokinetic data

Study	subject	form	dose	C_{max}	T_{max}	AUC_{0-t}	$AUC_{0-\infty}$	K_{el}	$T_{1/2}$
Unit			mg	ng/mL	H	h)*(ng/mL)	h)*(ng/mL)	1/h	H
S12PI	12	T	20	1.6642	4.67	20.9436	23.0778	0.030035	23.0783
S12PII	12	R	20	7.5354	4.33	70.7873	72.7891	0.067338	10.2935
S13PI	13	T	20	2.8641	4.33	44.6933	45.9192	0.052288	13.2563
S13PII	13	R	20	0.5901	4.33	8.36094	10.9241	0.025393	27.0855
S14PI	14	R	20	12.6207	4.33	152.935	155.516	0.041487	16.7075
S01 PI	1	R	20	2.0863	9	44.5254	46.7614	0.041414	16.7369
S14PII	14	T	20	6.2613	5	80.7585	83.5319	0.027728	24.9979
S01 PII	1	T	20	0.9243	5	18.5691	22.0531	0.017107	40.5183
S02PI	2	T	20	4.886	4.33	48.7595	51.0323	0.034804	19.916
S02PII	2	R	20	8.1366	3	87.5441	92.238	0.053986	12.8395
S03PI	3	T	20	3.5873	4.33	38.7068	41.8095	0.048376	14.3282
S03PII	3	R	20	0.827	4.33	7.01802	8.96286	0.081395	8.51587
S04PI	4	R	20	12.042	4.33	140.714	144.508	0.029396	23.58
S04PII	4	T	20	10.9047	4	155.724	158.29	0.038453	18.026
S05PI	5	R	20	1.6302	4.33	23.0624	24.3284	0.04052	17.1063
S05PII	5	T	20	1.8683	4.33	23.3967	26.1598	0.044335	15.6345
S06PI	6	T	20	5.2846	4.67	29.5536	32.073	0.044972	15.4129
S06PII	6	R	20	1.7713	2	14.5949	17.0464	0.032756	21.1612
S07PI	7	R	20	5.8779	5.67	63.2641	65.5054	0.06572	10.5469
S07PII	7	T	20	4.879	4.67	49.7936	51.0746	0.074707	9.27817
S08PI	8	T	20	14.3223	4.33	127.558	129.167	0.036612	18.9321
S08PII	8	R	20	18.4716	4.33	149.008	151.143	0.051486	13.463
S09PI	9	T	20	2.9133	4.33	29.7072	32.429	0.048534	14.2816
S09PII	9	R	20	6.8579	4.33	63.8033	67.107	0.060537	11.4499
S10PI	10	R	20	11.8206	4.67	147.252	194.546	0.03411	20.3208
S10PII	10	T	20	7.5661	5.33	104.448	106.55	0.036808	18.8313
S11PI	11	R	20	2.5976	4.67	37.4792	40.3531	0.040258	17.2175
S11PII	11	T	20	3.6917	4.33	68.2329	70.3878	0.032484	21.3381

Trospium pharmacokinetic data

Criteria for Bioequivalence

For Trospium chloride, based on the 90% confidence interval of the relative means C_{max}, AUC_{total} and AUC_{last} of the test product to the reference product should be between 80 and 125% for log transformed data. If 90% confidence interval for the difference of log – transformed AUC_{tot}, AUC_{last} and C_{max} lie within 80% and 125% then bioequivalence will be concluded.

No. of Subjects: 14

Table: 6.3 Pharmacokinetic statistical report

Parameters	**Reference Confidence Interval**	**90% standard confidence interval: [test form /ref form]**	**Result**
C_{max}	0.8 – 1.25	0.61741 – 1.4699	Cannot conclude equivalence
AUC_{0-t}	0.8 – 1.25	0.65451 – 1.5247	Cannot conclude equivalence
$AUC_{0-\infty}$	0.8 – 1.25	0.65364 – 1.4202	Cannot conclude equivalence

Since the confidence interval does not lie within the acceptance range of 80 to 125%, the Test and Reference Products are **not Bioequivalent** for TROSPIUM CHLORIDE**.**

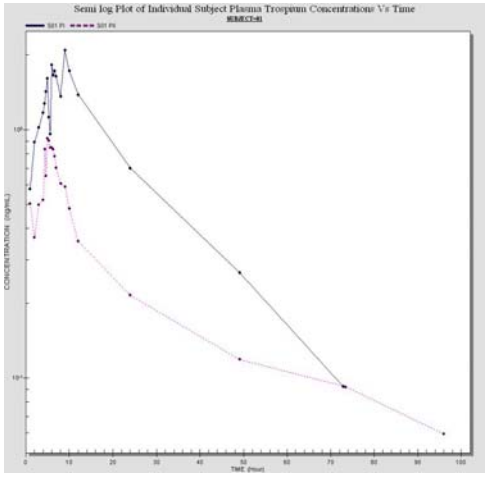
6.3 PHARMACOKINETIC GRAPHS:

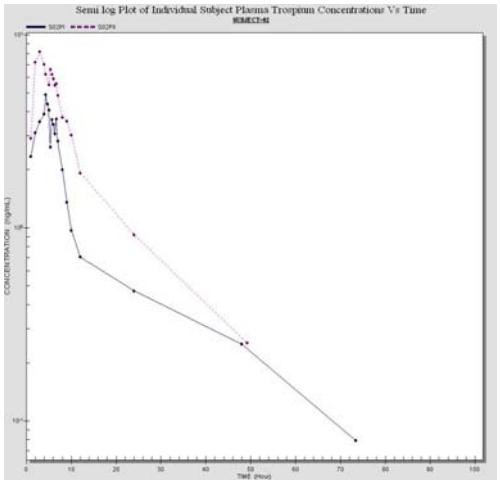
The pharmacokinetic graphs generated for individual subjects with the help of KINETICA^{tm} software are shown below: **Error! Bookmark not defined.**

Fig : 6.2 concentration Vs time graphs corresponding to S1,S2,S3,S4

Semi-long plots of individual subject plasma trospium :Concentration Vs time

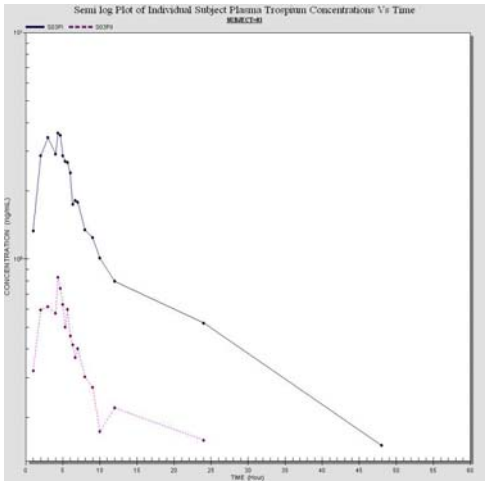
S1 S2





Semi-long plots of individual subject plasma trospium :Concentration Vs time

S3, S4



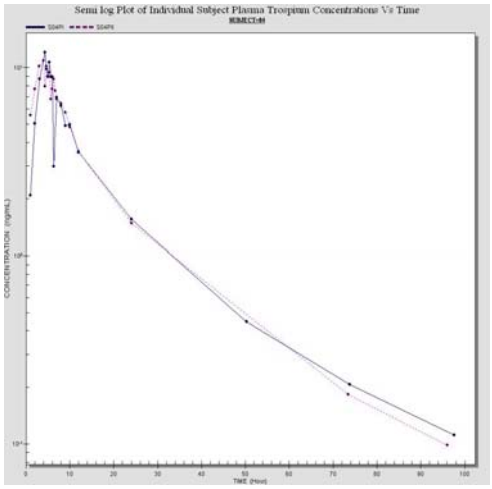
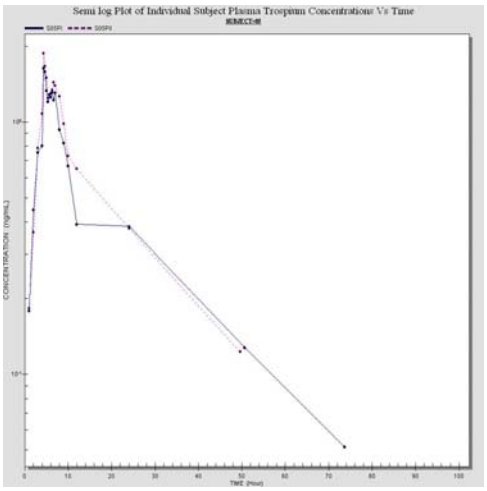
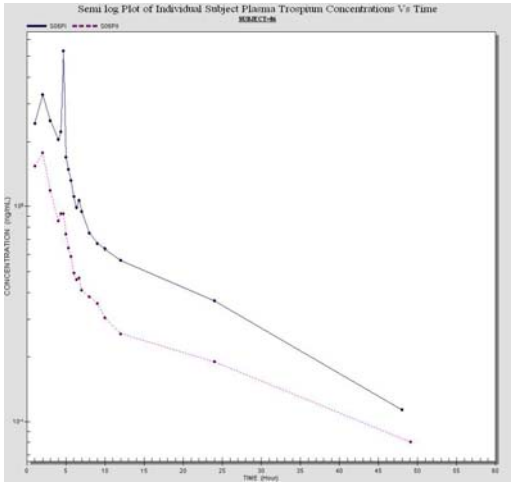


Fig :6.3 concentration Vs time graphs corresponding to S5,S6,S7,S8

Semi-long plots of individual subject plasma trospium :Concentration Vs time

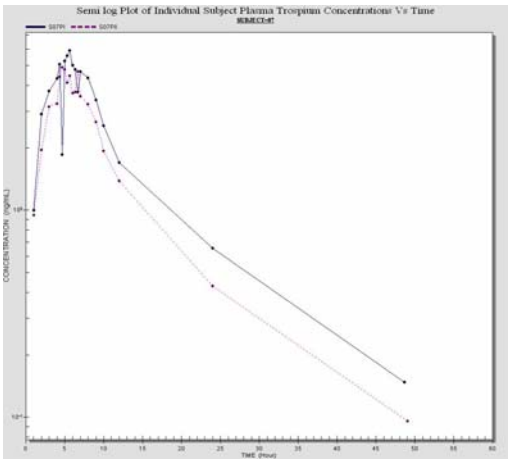
S5 S6





Semi-long plots of individual subject plasma trospium :Concentration Vs time

S7 S8



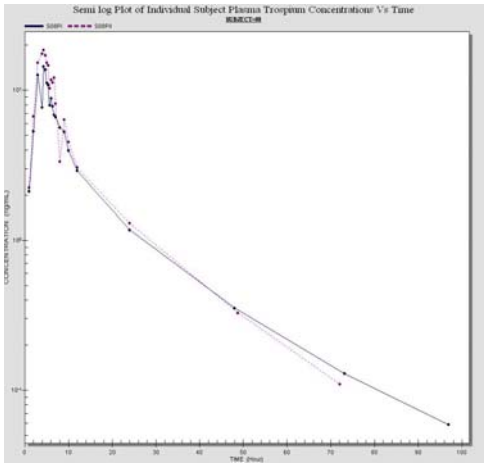
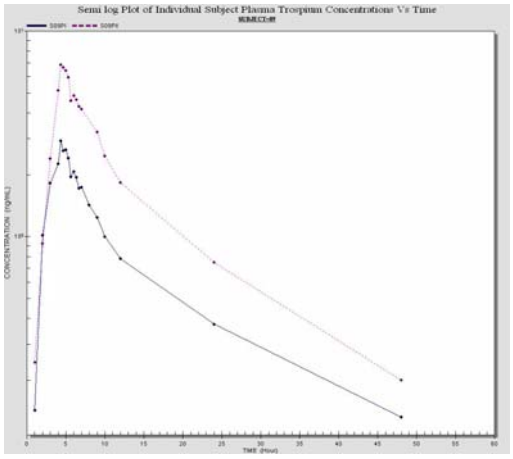
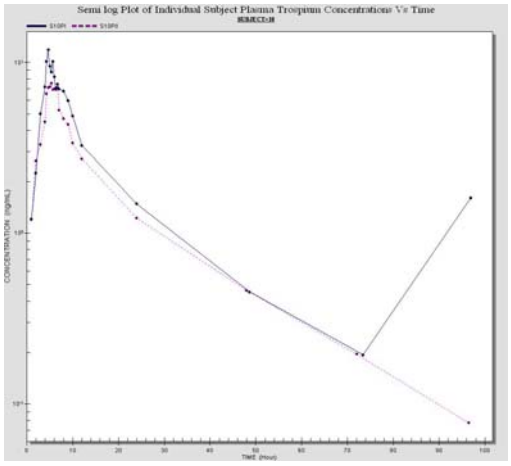


Fig :6.4 concentration Vs time graphs corresponding to S9,S10,S11,12

Semi-long plots of individual subject plasma trospium :Concentration Vs time

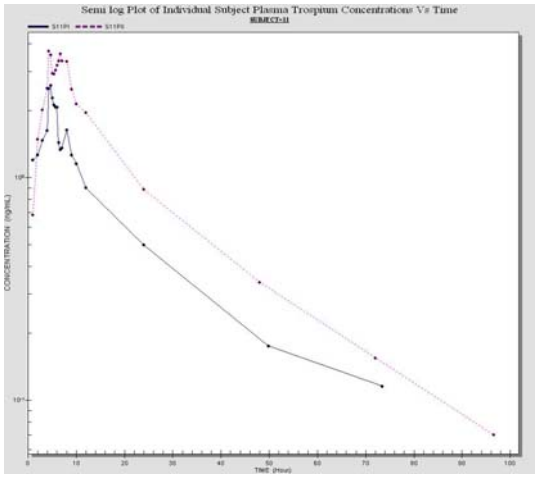
S9 S10

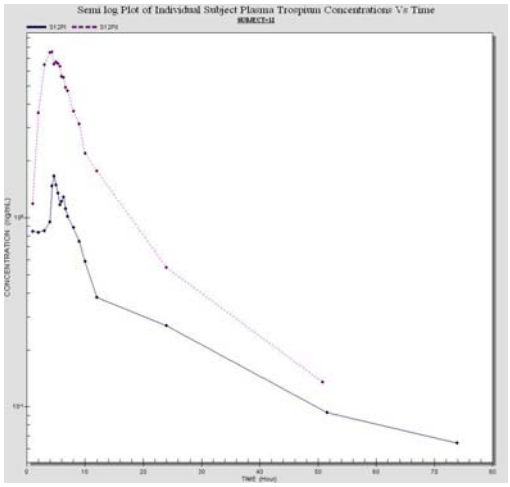




Semi-long plot of individual subject plasma trospium :Concentration Vs time

S11 S12

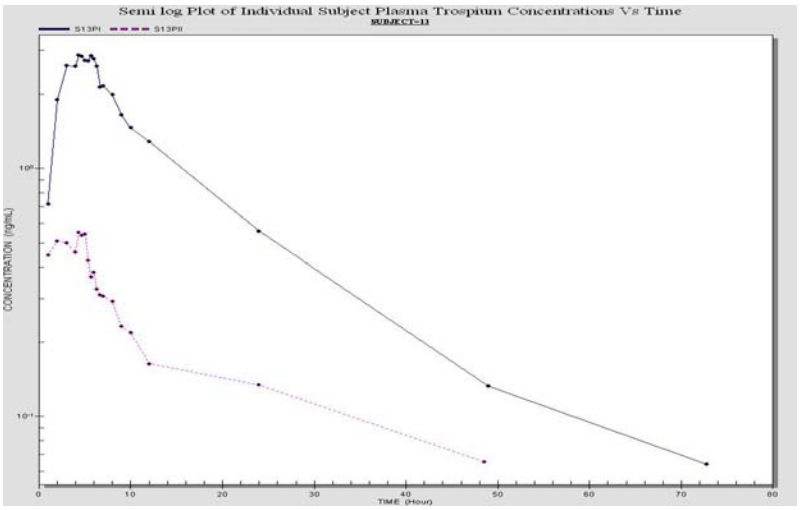




Semi-long plot of individual subject plasma trospium :Concentration Vs time

Fig 6.5 concentration Vs time graphs corresponding to S13,S14

S13



S14

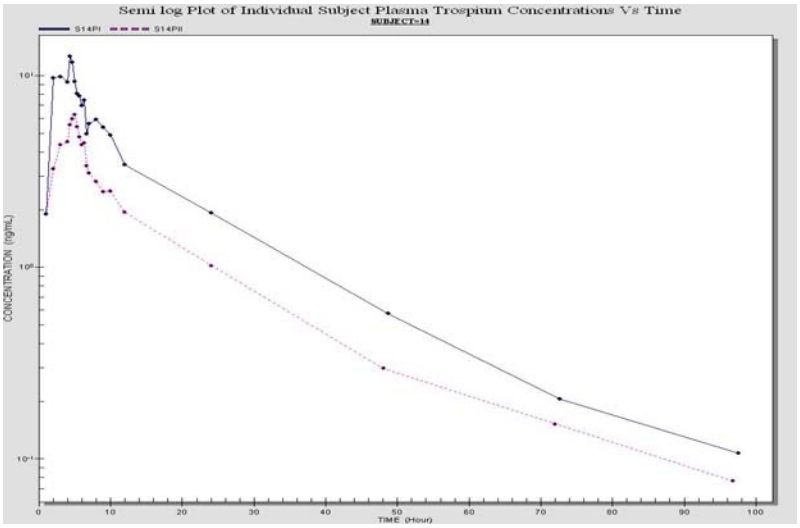


Fig:6.6 linear plot of trospium chloride conc vs time

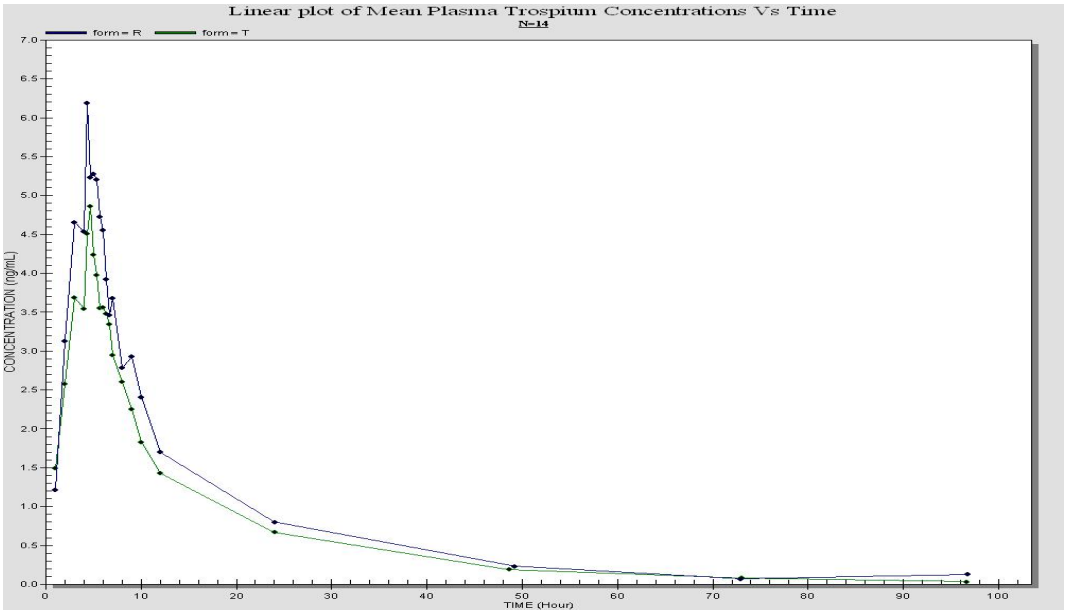
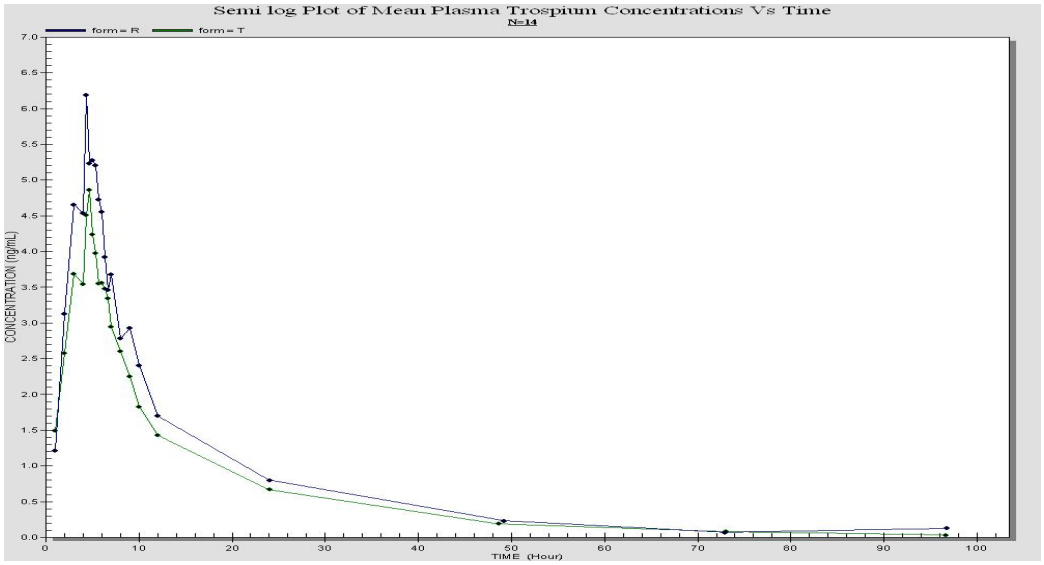


Fig 6.7: semi log plot of trospium chloride



DISCUSSION

The main objective of the trial was to evaluate the single-dose oral bioequivalence and to monitor the adverse event of test product Trospium chloride in healthy, adult, human subjects. The clinical study was conducted with healthy, adult, human male subjects under fasting conditions. The Subjects in the age group 18-50

years and who met the other study eligibility criteria were enrolled and completed the study.

The safety, pharmacokinetics and performance profiles of Trospium chloride Test product was evaluated and compared with those of the commercialized reference formulation of Trospium chloride. The effect of Trospium chloride under oral administration to 14 healthy human male subjects was safe and equally well tolerated. Bio-analytical data were available after analysis of the samples at the Bio-analytical Department.

The PK Analysis result indicates that the 90% confidence interval does not lie within the acceptance range of 80 to 125% for Trospium chloride.

From the above results and PK analysis the reference and test product of the Trospium chloride is considered to be biologically not equivalent. There are so many factors played a very important role in the present bioavailability study. These factors include No of subjects ,Dose, Sampling time points, Randomization Protocol deviations, formulation variation not only these every parameter have its importance in BA/BE studies and finally all these parameters play a key role in final result.

CHAPTER-7

CONCLUSION

The safety, pharmacokinetics and performance profiles of trospium test product was evaluated and compared with those of the commercialized reference formulation of trospium chloride. The effect of trospium chloride under oral administration to 14 healthy human male subjects was safe and equally well tolerated. Based on the PK results, the 90% confidence interval does not lie within the acceptance range of 80 – 125% for trospium. The reference and test product

of the drug is considered biologically not equivalent since they resulted in highly variable rates and extents of exposure of trospium. Overall, the test formulation of trospium chloride has a number of disadvantages over the conventional reference formulation of trospium Chloride, since the test formulation of trospium chloride resulted in highly variable pharmacokinetic profiles to the reference formulation. After a single dosage, the two formulations are expected to result in highly variable efficacy for the treatment of urinary incontinence. Therefore it can be concluded that test and reference products are biologically not equivalent under fasting conditions.

CHAPTER-VIII

APPENDIX-I

ACTIVITIES SCHEDULE

Study phase	**Screening** With in 28 days of dosing	Check-in day	Dosing day
Activity			

Informed consent	X	X	
Medical history and demographic data	X		
Physical examination	X		
ECG (12-lead)	X		
Vital signs measurement	X		
Hematology	X		
Urine analysis	X		
Clinical chemistry	X		
Serology(HIV-I&II, hepatitis B&C, RPR)	X		
Urine drug screening ethanol, benzodiazepines, cannabionoids, amphetamines, cocaines, opiates (period-I)		X	
Record of concomitant medication		X	X
Check in procedures		X	
Alcohol screening(periods I&II)	X	X	
Confinement in study unit		X	X
Drug dosing			X
PK blood sampling			X
Adverse events monitoring		X	X

APPENDIX-II

Pre-Study Laboratory Safety Evaluation Parameters

Clinical chemistry

S.No.	PARAMETER	REFERENCE INTERVALS	UNITS
1	Random blood glucose	70 to170	mg/dl
2	Urea	15 to 40	mg/dl
3	Creatinine	0.6 to 1.5	mg/dl
4	Total bilirubin	0 to 1.0	mg/dl
5	SGPT(ALT)	30 to 65	U/L
6	SGOT(AST)	15 to 37	U/L
7	Alkaline phosphatase(ALP)	50 to 136	U/L
8	Albumin	3.7 to 5.3	g/dl
9	Sodium	136 to149	mEq/L
10	Potassium	3.8 to 5.2	mEq/L

SEROLOGY

SEROLOGY SCREENING	INDICATION
HIV-1&2	HIV ANTIBODIES AIDS
Abs antigen& HCV	HEPATITS B& HEPATITIS C VIRUS
RPR Test	SYPHILIS

HAEMATOLOGY

S.No.	PARAMETER	REFERENCE INTERVALS	UNITS
1	Total W.B.C	4000 to 1100	/cmm
2	Total R.B.C	4.5 to 6.5	mil/cu mm
3	Hemoglobin	14 to 18	gms%
4	HCT(PCV)	40 to 54	Vol%
5	Neutrophils	40 to 75	%
6	Lymphocytes	20 to 40	%
7	Eosinophils	01 to 06	%
8	Monocytes	2 to 8	%
9	Basophils	0 to1	%
10	Platelets	104 to 400 x 10^3	/cmm
11	ESR(1^{st} hr)	0 to 15	Mm

URINE ANALYSIS

PARAMETER
Physical examination
Color
Transparancy
Specific gravity
Ph
Chemical examination
Glucose
Protein
Ketones
Blood
Urobilinogen
Bilirubin
Microscopical examination
Pus cells
RBC’s
Epithelial cells
Crystals
Casts
Others

APPENDIX-III

MEAL MENU FOR SUBJECTS PARTAKING

IN THE PRIMIDONE STUDY

Pre-study dinner	(Standard meal)Rice with one curry, dal, sambar, rasam, curd and papad
Break fast	----------
Lunch	Standard meal
Snacks	Two somosa
Dinner	Standard meal

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